PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 4 :
C07K 13/00, 15/12, 15/14
C12N 1/18, 15/00, C12P 19/34
C12P 21/00, 21/02, C07H 21/04
// (C12P 19:34, C12R 1:91)
(C12P 21/02, C12R 1:865)

A1

(11) International Publicati n Number: **WO 88/ 07548**

(43) International Publication Date: 6 October 1988 (06.10.88)

(21) International Application Number: PCT/AU88/00093

(22) International Filing Date: 31 March 1988 (31.03.88)

(31) Priority Application Numbers: PI 1209
PI 3317
PI 4903
PI 6005

(32) Priority Dates: 2 April 1987 (02.04.87)
24 July 1987 (24.07.87)
15 October 1987 (15.10.87)
21 December 1987 (21.12.87)

(33) Priority Country: AU

(71) Applicant *(for all designated States except US)*: AMRAD CORPORATION LIMITED [AU/AU]; Unit 6, 663 Victoria Street, Abbotsford, VIC 3067 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : GEARING, David, Paul [GB/AU]; 134 Miller Street, North Fitzroy, VIC 3068 (AU). GOUGH, Nicholas, Martin [GB/AU]; 20 Rangeview Grove, North Balwyn, VIC 3104 (AU). HILTON, Douglas, James [AU/AU]; 8 West End Road, Warrandyte, VIC 3113 (AU). KING, Julie, Ann [AU/AU]; 121 Springvale Road, Nunawading, VIC 3131 (AU). METCALF, Donald [AU/AU]; 268 Union Road, Balwyn, VIC 3103 (AU). NICE, Edouard, Collins [GB/AU]; 9 Odessa Street, St. Kilda, VIC 3182 (AU). NICOLA, Nicos, Anthony [AU/AU]; 59 Queen Street, Regent, VIC 3073 (AU). SIMPSON, Richard, John [AU/AU]; 42 Stanley Street, Richmond, VIC 3121 (AU). WILLSON, Tracy, Ann [AU/AU]; 26 Fortuna Avenue, North Balwyn, VIC 3104 (AU).

(74) Agents: SLATTERY, John, Michael et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AU, BR, DK, FI, HU, JP, KR, NO, US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: LEUKAEMIA-INHIBITORY FACTOR

(57) Abstract

A leukaemia-inhibitory factor (LIF) is disclosed, together with a method of preparation of LIF in essentially pure form. Nucleotide and amino acid sequences are disclosed, together with recombinant DNA molecules and host cells for production of polypeptides having LIF activity.

```
                                  ###          ###      •••
HisTrpLysHisGlyAlaGlySerProLeuProIleThrProValAsnAl   17
CACTGGAAACACGGGGCAGGGAGCCCTCTTCCCATCACCCCTGTAAATGC   50

aThrCysAlaIleArgHisProCysHisGlyAsnLeuMetAsnGlnIleL   34
CACCTGTGCCATACGCCACCCATGCCACGGCAACCTCATGAACCAGATCA   100

                      •••
ysAsnGlnLeuAlaGlnLeuAsnGlySerAlaAsnAlaLeuPheIleSer   50
AGAATCAACTGGCACAGCTCAATGGCAGCGCCAATGCTCTCTTCATTTCC   150

TyrTyrThrAlaGlnGlyGluProPheProAsnAsnValGluLysLeuCy   67
TATTACACAGCTCAAGGAGAGCCGTTTCCCAACAACGTGGAAAAGCTATG   200
        •••  ###                  •••
sAlaProAsnMetThrAspPheProSerPheHisGlyAsnGlyThrGluL   84
TGCGCCTAACATGACAGACTTCCCATCTTTCCATGGCAACGGGACAGAGA   250

ysThrLysLeuValGluLeuTyrArgMetValAlaTyrLeuSerAlaSer   100
AGACCAAGTTGGTGGAGCTGTATCGGATGGTCGCATACCTGAGCGCCTCC   300
    •••                      •••  ###
LeuThrAsnIleThrArgAspGlnLysValLeuAsnProThrAlaValSe   117
CTGACCAATATCACCCGGGACCAGAAGGTCCTGAACCCCACTGCCGTGAG   350
                •••
rLeuGlnValLysLeuAsnAlaThrIleAspValMetArgGlyLeuLeuS   134
CCTCCAGGTCAAGCTCAATGCTACTATAGACGTCATGAGGGGCCTCCTCA   400

erAsnValLeuCysArgLeuCysAsnLysTyrArgValGlyHisValAsp   150
GCAATGTGCTTTGCCGTCTGTGCAACAAGTACCGTGTGGGCCACGTGGAT   450

ValProProValProAspHisSerAspLysGluAlaPheGlnArgLysLy   167
GTGCCACCTGTCCCCGACCACTCTGACAAAGAAGCCTTCCAAAGGAAAAA   500

sLeuGlyCysGlnLeuLeuGlyThrTyrLysGln                   178
GTTGGGTTGCCAGCTTCTGGGGACATACAAGCAAG                  535
```

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark	MC	Monaco	US	United States of America
FI	Finland	MG	Madagascar		

LEUKAEMIA INHIBITORY FACTOR

FIELD OF THE INVENTION

This invention relates to Leukaemia Inhibitory Factor (LIF), to the production of LIF in substantially pure form, and to the cloning and expression of recombinant LIF.

BACKGROUND OF THE INVENTION

Mature blood cells are produced by the clonal proliferation and concomitant differentiation of immature precursor cells (Metcalf,D. (1984) The Hemopoietic Colony Stimulating Factors, Elsevier, Amsterdam.). For normal haemopoietic cells, the two processes of proliferation and differentiation are tightly coupled so that the short-lived mature cells are continually replenished. At least four biochemically distinct growth factors, the colony-stimulating factors (CSFs), have been shown in vitro to stimulate the proliferation and differentiation of precursor cells of granulocytes and macrophages: G-CSF, M-CSF, GM-CSF and Multi-CSF (IL-3) (see Metcalf, D.

(1987), Proc.R.Soc.B. 230, 389-423, for review).

In contrast to normal cells, leukaemic myeloid cells are characterized by an uncoupling of proliferation and differentiation so that immature progenitor cells accumulate, retain their proliferative capacity and fail to differentiate. There has been considerable controversy concerning the nature of biological factors that are able to induce the differentiation of myeloid leukaemic cells in vitro and whether certain factors are capable of inducing differentiation in the absence of proliferation. Indeed, it has been proposed that the events of proliferation and differentiation are necessarily mediated by different factors (Sachs, L. (1982), J.Cell.Physiol.Suppl., 1, 151-164). On the one hand, two groups have described and purified an activity (MGI-2 or D-factor) capable of inducing the differentiation of the murine myeloid leukaemic cell line M1, that does not stimulate the proliferation of normal progenitor cells (Lipton, J.H. and Sachs, L. (1981), Biochem.Biophys. Acta. 673, 552-569; Tomida, M., Yamamoto-Yamaguchi, Y., and Hozumi, M. (1984) J.Biol.Chem. 259, 10978-10982). On the other hand, the present inventors have previously shown that one of the CSFs, G-CSF, is a strong differentiation-inducing stimulus for the murine myeloid leukaemic cell line, WEHI-3B D^+, as well as being a proliferative and differentiative stimulus for normal cells (Nicola,N.A., Metcalf, D., Matsumoto, M. and Johnson, G.R. (1983), J.Biol.Chem. 258, 9017-9023). Moreover, Tomida, et.al. (Tomida, M., Yamamoto-Yamaguchi, Y., Hozumi, M., Okabe, T. and Takaka, F. (1986), FEBS Lett., 207, 271-275) have shown that recombinant G-CSF is also able to induce the macrophage differentiation of M1 cells. The relationship between these factors has been a matter of debate. The situation was further complicated

by the finding that tumour necrosis factor α(TNFα) is capable of stimulating the differentiation of the human myeloblastic cell line ML-1 (Takeda,K., Iwamoto, S., Sugimoto, H., Takuma,T., Kawatani,N., Noda,M., Masaki,A., Morise,H., Arimura,H. and Konno,K. (1986) Nature 323, 338-340).

In an attempt to resolve the discrepancies between the data of these groups, the present inventors have biochemically fractionated the medium conditioned by Krebs II ascites tumour cells used in a number of previous studies and shown it to contain not only authentic G-CSF and GM-CSF, active on normal progenitor cells as well as WEHI-3B D^+ cells, but also two biochemically distinct, but functionally similar, factors capable of inducing the differentiation of M1 cells. These latter factors have been termed leukaemia inhibitory factor (LIF)-A and LIF-B because of their ability to suppress the proliferation of M1 leukaemic cells in vitro and it has been shown that they do not induce the differentiation of WEHI-3B D^+ cells and do not stimulate the proliferation of normal granulocyte/macrophage progenitor cells.

SUMMARY OF THE INVENTION

LIF, in accordance with the present invention, is defined as a molecule that has the following two properties: (1) it has the ability to suppress the proliferation of myeloid leukaemia cells such as M1 cells, with associated differentiation of the leukaemic cells; and (2) it will compete with a molecule having the defined sequence of murine LIF or human LIF hereein for binding to specific cellular receptors on M1 cells or murine or human macrophages. LIF, has the potential for use as a therapeutic non-proliferative agent for suppressing some forms of myeloid leukaemia as well as a reagent for modifying macrophage function and other responses to

infections, and clinical testing and research studies relating to these.

The term "polypeptide having LIF activity" as used herein denotes a polypeptide or glycopolypeptide having the properties of LIF as defined above, including but not restricted to polypeptides having an amino acid sequence which is fully or partially homologous with the amino acid sequence of either murine or human LIF as disclosed herein. This term also includes polypeptides which are fully or partially homologous with a portion only of the amino acid sequence of murine or human LIF provided that the polypeptide has the properties of LIF as defined above. This term further includes polypeptides or glycopolypeptides produced by expression in a host cell which are inactive when expressed but which are processed by the host cell to yield active molecules, as well as polypeptides or glycopolypeptides which are inactive when expressed but which are selectively cleavable <u>in vitro</u> or <u>in vivo</u> to yield active molecules.

Murine LIF is a molecule having the following biological properties:

(a) induction of macrophage differentiation in cells of the murine myeloid leukaemic cell line M1 with loss of proliferative capacity and death of the clonogenic leukaemic cells, an action potentiated by G-CSF;

(b) selective binding to high affinity receptors on M1 cells and on normal murine monocytes and macrophages from the peritoneal cavity, spleen and bone marrow, with the number of receptors increasing with macrophage maturation or functional activation, but not to granulocytic, erythroid or lymphoid cell from these tissues;

(c) specific binding of ^{125}I-LIF to high affinity

receptors is not competed for by G-CSF, GM-CSF, Multi-CSF (interleukin-3), M-CSF, interleukins 1,2, 4 or 6, endotoxin or a variety of other growth factors, but is competed for by unlabeled LIF.

(d) elevation by bacterial endotoxin in the serum of normal or athymic mice, but not in endotoxin-resistant C3H/HeJ mice;

(e) production by various tissues including lung, salivary gland, peritoneal cells and bone shaft;

(f) reduction of the survival time in vitro of normal granulocyte-macrophage progenitor cells when grown in the absence of CSF;;

(g) an inability to suppress proliferation or induce differentiation of WEHI-3B D^+ murine myeloid leukaemic cells, murine myeloid cells transformed to leukaemogenicity by infection with retroviruses expressing GM-CSF or Multi-CSF, the murine leukaemic cell lines WEHI265 and WR19;

(h) no ability to stimulate the proliferation of normal progenitor cells of the granulocyte, macrophage, eosinophil, megakaryocyte, erythroid and mast cell lineages, and an inability to suppress the clonal proliferation or alter the quantitative responsiveness to stimulation by CSFs in vitro of progenitors of normal granulocytes, macrophages, megakaryocytes, eosinophils or natural cytotoxic cells or the proliferation of cells of the continuous cell lines 32CD1.13 and FDCP-1;

(i) an inability to compete with the iodinated derivatives of granulocyte colony-stimulating factor (G-CSF) for binding to specific cellular receptors despite the ability of G-CSF to induce

differentiation in M1 and WEHI-3B D^+ murine myeloid leukaemic cells and to potentiate the action of LIF on M1 cells;

(j) an inability of the action of LIF to be inhibited by antisera specifically raised against tumour necrosis factor (TNF);

(k) transcripts for murine LIF are present constitutively in the cytoplasm of LB3 and E9.D4 T cells, are not induced by the lectin concanavalin A in these cells, and are present in a number of other cell types;

Murine LIF has also been determined to have the following properties:

(l) a single subunit glycoprotein with molecular weight of 58,000± 5,000 as determined by electrophoresis in 8-25% gradient polyacrylamide gels containing sodium dodecyl sulphate with or without reducing agent (2-mercaptoethanol or dithiothreitol), and a molecular weight of 23,000 ± 5,000 after treatment with the endoglycosidase, N-glycanase;

(m) an isoelectric point between 8.6 and 9.3;

(n) the primary amino acid sequence detailed in Figures 10, 11 and 15 herein;

(o) is encoded by the nucleotide sequence detailed in Figures 10, 11 and 15 herein;

(p) a specific activity of 1-2 x 10^8 units/mg (where 50 units are defined as the amount of LIF which in one millilitre induces a 50% reduction in the clone formation by murine M1 myeloid leukaemic cells);

(q) is encoded by a unique gene on murine chromosome 11 (as determined by analysis of a panel of mouse/Chinese hamster ovary somatic cell hybrids)

bounded by restriction endonuclease cleavage sites as illustrated in Figure 19 herein;

(r) is homologous to a human gene sequence bounded in chromosomal DNA by restriction endonuclease cleavage sites as illustrated in Figure 27 herein.

Human LIF is a molecule having the following biological properties of the native and/or yeast derived recombinant product:

(a) induction of macrophage differentiation in cells of the murine myeloid leukaemia cell line M1, with loss of proliferative capacity and death of the clonogenic leukaemia cells;

(b) no ability to stimulate the proliferation of normal human progenitor cells of the granulocyte, macrophage, eosinophil and erythroid lineages;

(c) an ability in combination with G-CSF to partially suppress the proliferation of cells of the human leukaemic cell line U937 and in combination with GM-CSF the proliferation of the human leukaemic cell lines U937 and HL60;

(d) binds specifically to murine LIF receptors on M1 cells and murine macrophages and competes completely for the binding of murine ^{125}I-LIF to such cells.

(e) binds to specific cellular receptors on the human hepatoma cell line Hep-2G.

Human LIF has also been determined to have the following properties:

(f) is identical to murine LIF at approximately 80% of amino acid residues in the mature protein;

(g) the primary amino acid sequence detailed in Figures 25, 26 and 29 herein;

(h) is encoded by a unique gene bounded in chromosomal DNA by restriction endonuclease

cleavage sites as illustrated in Figure 27 herein;

(i) has as a portion of the sequence of its gene, the nucleotide sequence detailed in Figure 25 herein.

In a first aspect of the present invention, there is provided leukaemia-inhibitory factor (LIF), in essentially pure form. This invention also provides methods for the production of essentially pure LIF, particularly for the production of essentially pure murine LIF by purification from Krebs II ascites tumour cells, and of essentially pure human LIF by purification from the human bladder carcinoma cell line 5637 (ATCC No. HTB 9). Essentially pure LIF may also be produced by host cells, such as yeast cells, mammalian cells and E.coli, containing recombinant DNA molecules coding for the amino acid sequence of LIF, or part thereof

The references herein to "in essentially pure form" denote a form of polypeptide or glycopolypeptide in which at least 90% of the polypeptide or glycopolypeptide appear as a single band when electrophoresed on an appropriate sodium dodecyl sulphate polyacrylamide gel, said band being coincident with LIF activity.

In another aspect there is provided a method for isolation of recombinant DNA clones containing nucleotide sequences encoding the LIF protein, either completely or in part. The present invention also relates to a nucleotide sequence which has the capacity to encode the unique sequence of amino acids determined to be characteristic of LIF and to furthermore allow the complete amino acid sequence of LIF to be inferred.

In yet another aspect, this invention extends to recombinant DNA molecules containing the aforementioned nucleotide sequences encoding LIF (or substantially similar analogues thereof), either completely or in part, in a form in which said nucleotide sequences are able to

direct the synthesis and production of LIF, either completely or in part. This aspect of the invention also extends to cloning vectors (such as plasmids) and host cells having such recombinant DNA molecules inserted therein. Furthermore, the invention also extends to synthetic LIF, either complete or in part, or substantially similar analogues thereof, produced by expression of such recombinant DNA molecules or by peptide synthesis.

DETAILED DESCRIPTION OF THE INVENTION

In the present investigation of LIF, in particular with a view to providing alternative sources of this glycoprotein of both murine and human origin to enable use in clinical and other applications, murine LIF has been purified from Krebs II ascites tumour cells by an efficient purification procedure and has been subjected to partial amino acid sequence analysis as a first step towards chemical or biosynthetic production of this factor.

LIF radioactively derivatized with I^{125} has been used in a receptor competition assay to identify cells and tissues of murine and human origin that synthesize and produce LIF. As a result of this survey, a recombinant DNA library of DNA copies of mRNA from one of these sources has been screened and murine LIF-encoding clones identified by hybridization with radioactively-labelled oligonucleotides encoding portions of the previously determined murine LIF amino acid sequence, and the aforementioned murine LIF cDNA clones subjected to nucleotide sequence analysis. Moreover, the cloned murine LIF encoding sequence has been used as a hybridization probe to identify a human gene encoding a homologue of murine LIF and hybridization conditions established under which said cross-species hybridization can be effectively performed. As a result, recombinant DNA clones containing

DNA sequences encoding the human homologue of murine LIF have been identified.

As a result of this construction of LIF- encoding sequences, recombinant DNA molecules have been constructed that use cloned murine or human LIF- encoding DNA sequences to direct the synthesis of clonally pure LIF, either completely or in part, for example in cultured mammalian cells, in yeast or in bacteria. This invention thus extends to substantially pure LIF produced in this manner.

In one particular embodiment, this invention relates to the expression of the cloned human and murine LIF genes in yeast cells, in fibroblasts and in E.coli, and to the modification of the cloned gene so that it may be so expressed, as well as to the establishment of the biological and biochemical properties of recombinant human and murine LIF.

In this embodiment also, the present invention relates to recombinant DNA molecules containing nucleotide sequences encoding human or murine LIF (or substantially similar analogues thereof), either completely or in part, in a form in which said nucleotide sequences are able to direct the synthesis and production of human or murine LIF in yeast cells, fibroblasts or E.coli, either completely or in part. This invention also provides cloning vectors (such as plasmids) and host cells having such recombinant DNA molecules inserted therein. Furthermore, the invention extends to synthetic human or murine LIF, either complete or in part, or substantially similar homologues thereof, produced by expression of such recombinant DNA molecules in yeast cells. In one embodiment of the present work relating to LIF, the human and murine LIF gene sequences have been modified and installed in a yeast expression vector, YEpsecl. Yeast cells have been

transformed with the resulting recombinants and the medium conditioned by yeast cells so transformed has been shown to contain a factor with biological properties analogous to those of native human and murine LIF.

In view of the potential of LIF for use in the treatment of patients with some forms of myeloid leukaemia and patients with certain infections, the present invention also extends to pharmaceutical compositions comprising LIF, particularly human LIF, either completely or in part, produced for example using cloned LIF-encoding DNA sequences or by chemical synthesis, and to pharmaceutical compositions of analogues of LIF, for example produced by chemical synthesis or derived by mutagenesis of aforesaid cloned LIF-encoding DNA sequences. The pharmaceutical compositions may also contain at least one other biological regulator of blood cells, such as G-CSF or GM-CSF. Furthermore, the invention also extends to diagnostic reagents for use in detecting genetic rearrangements, alterations or lesions associated with the human LIF gene in diseases of blood cell formation, including leukaemia and congenital diseases associated with susceptibility to infection.

EXAMPLE 1

The accompanying Figures 1 to 5 relate to various steps of the purification method described below, showing where LIF is pooled in each fractionation step and showing evidence of its purity. In the Figures:

Figure 1: relates to Step 2 of the purification of LIF on DEAE-Sepharose CL-6B. The bar indicates fractions that are pooled for Step 3.

Figure 2: relates to Step 3 of the purification of LIF on Lentil lectin Sepharose 4B. The bar indicates fractions that are pooled for Step 4.

Figure 3: relates to Step 4 of the purification of LIF on CM-Sepharose CL-6B. The bar indicates fractions that are pooled for Step 5.

Figure 4: relates to Step 5 of the purification of LIF on a fatty acid analysis HPLC column. Fractions from 39.6 to 41.3% acetonitrile are pooled.

Figure 5: shows the molecular weight and purity of purified LIF. The upper panel shows the migration of protein standards (93,000; 67,000; 43,000; 30,000; 20,000 and 14,000 molecular weight) and purified LIF (two preparations) on 8-25% polyacrylamide SDS gel. The protein and biological activity are both associated with a protein of molecular weight 58,000.

Step 1 Krebs II tumour cells (1×6^6) are injected intraperitoneally into C57Bl6/6fj/ WEHI mice and after 7 days the mice are sacrificed and the peritoneal fluid removed. The cells are centrifuged, resuspended at 5×10^6 cells/ml in Dulbecco's modified Eagle's medium containing E.coli lipopolysaccharide (200ng/ml) and incubated for 24 hrs at 37°C in a humidified incubator containing 10% CO_2 in air. The cells are again centrifuged, the conditioned medium removed, made up to 0.02% (w/v) sodium azide and stored at 4°C. Thirty-six litres of conditioned medium are concentrated to 100ml using a HIP10-8 hollow fibre cartridge in an Amicon DC2A concentrator, exchanged into 10mM Tris-HCl buffer pH8.0 containing 1mM phenylmethyl sulphonyl fluoride (PMSF), sodium azide (0.02% w/v) and Tween 20 (0.02%v/v) and re-concentrated to 20ml over a YM-10 membrane in an Amicon stirred cell.

Step 2. The concentrate is applied to a column (2.5 x 30cm) of DEAE-Sepharose CL-6B (Pharmacia) equilibrated in

the same buffer and eluted with 200ml of equilibration buffer followed by a 400ml linear gradient to 0.3M NaCl in the same buffer. The flow rate is 25ml/hr and 5.8ml fractions are collected and assayed. (See Fig.1)

Step 3. The fractions of protein which failed to bind to the gel during the pre-gradient step and which contained activity on murine Ml cells are pooled, concentrated over a YM-10 membrane, exchanged into 100mM sodium acetate buffer pH6.0 containing 1mM $MgCl_2$, 1mM PMSF, 0.2% sodium azide and 0.02% Tween 20 and applied to a column (1 x 4cm) of Lentil lectin-sepharose 4B (Pharmacia) equilibrated in the same buffer. The column is eluted with 25ml of equilibration buffer and then a 100ml linear gradient to 0.5 M α-methyl-D-mannopyranoside in the same buffer at a flow rate of 10ml/hr. Fractions of 3ml are collected and assayed. (See Fig.2)

Step 4. The fractions of proteins which bind to the gel and are eluted by the sugar and have activity on Ml cells are pooled, concentrated and exchanged into 100mM sodium acetate buffer pH5.0- containing 1mM PMSF, 0.02% sodium azide and 0.02% Tween 20 and applied to a column (1.0 x 4.0cm) of CM-Sepharose CL-6B (Pharmacia) equilibrated in the same buffer. The column is eluted with 25ml equilibration buffer and then a 100ml linear gradient to 1.0m NaCl in the same buffer and then by 25ml of 1.0M NaCl adjusted to pHl0 with NaOH. The flow rate is 2.0 ml/hr and 3.0ml fractions are collected. (see Fig.3)

Step 5. The fractions of proteins which bind to the gel and are eluted by the NaCl gradient and have activity on Ml cells are pooled, concentrated to 2ml over a YM-10 membrane and exchanged into 20mM ammonium acetate buffer

pH5.0 containing 0.02% Tween 20 and then filtered through a 0.45µ Millipore filter. The pool is loaded into the injector of a high performance liquid chromotography system (Beckman) equipped with a Waters fatty acid analysis column and dual pumps with gradient programmer. The column is equilibrated in water containing 0.1% (v/v) trifluoroacetic acid (TFA), the sample is injected, and the column is then eluted with a 5min linear gradient to 34.8% (v/v) acetonitrile in water and 0.1% TFA and then eluted with a 50min linear gradient to 49.8% (v/v) acetonitrile in water and 0.1% TFA. The flow rate is 1 ml/min and 1ml fractions are collected into polypropylene tubes containing 50µl of 100mM ammonium bicarbonate and 0.4% Tween 20. (see Fig.4)

The fractions with activity on M1 cells from step 5 consisted of two overlapping ultraviolent- absorbing peaks. Each peak was associated with activity on M1 cells and each peak corresponded to a single protein band of Mr 58,000 on sodium dodecyl sulphate polyacrylamide gels using silver staining. In each case, the protein band of Mr 58,000 contained the biological activity on M1 cells as assessed by cutting a parallel track on the gel (also loaded with LIF) into 1mm strips, extracting the protein from the gel strips and assaying the extracted protein for its ability to suppress proliferation and induce differentiation in M1 cells in semi-solid agar cultures. (see Fig.5)

EXAMPLE 2

The following Example sets out the steps used in obtaining the N-terminal amino acid sequence and amino acid sequences of various peptides generated by proteolytic cleavage with trypsin or Staphylococcal V8 protease.

LIF purified by the procedure of Example 1 (15µg) was applied to a microbore (2mm) column packed with Brownlee RP300 beads (C8) and eluted on a HPLC system with a linear gradient from 0-60% acetonitrile in 0.1% trifluoroacetic acid. The protein eluted as a single peak in a volumn of 100µl. This was applied to a glass fibre disc and allowed to dry and then placed in the sequencing chamber of an Applied Biosystem (Model 470A) gas phase protein sequencer. The protein was sequenced by Edman chemistry and the phenylthiohydantoin derivatives of the individual amino acids identified by HPLC on an Applied Biosystems 120A PTH analyser.

A separate aliquot of LIF (20µg) also purified by the aforementioned procedure, was diluted to 2ml in 6m guanidine hydrochloride, 0.2M Tris-HCL buffer pH8.5, 2mM dithiothreitol and incubated at 37°C for 2 hrs. Then a 30-fold molar excess of iodoacetic acid was added and the solution incubated at 37°C for a further 15min. The solution was applied to the same microbore HPLC column and eluted as described above. The reduced and carboxy-methylated derivative of LIF (RCM-LIF) eluted 3min later from the column than the untreated LIF and RCM-LIF (200µl) was diluted to 1ml with 0.1 M Tris-HCL buffer pH8.0 containing 0.02% Tween 20, 1mM $CaCl_2$ and 2µg of TPCK-treated trypsin. This incubation was allowed to proceed for 18hr at 37°C and the mixture was again applied to the microbore column and eluted in the same way as described above. Individual peptides appeared as UV-absorbing peaks, and were collected individually. Some of these peptides were sequenced directly as described above while others were repurified on the same column but using an 0-60% acetonitrile gradient in 0.9% NaCl pH6.0 before being sequenced.

A second aliquot of RCM-LIF (200µl=10µg)

was diluted to 1ml with 0.05M sodium phosphate buffer, pH 7.8 containing 0.002M EDTA and 0.02% Tween 20, and was digested with 2µg of Staphylococcus aureus V8 protease for 18 hours at 37°C. The reaction mixture was applied to the same microbore column and eluted as described above.

The 26 amino acids defining LIF from the amino terminal in sequence were found to be:

N-Pro-Leu-Pro-Ile-Thr-Pro-Val-X-Ala-Thr-X-Ala-Ile-Arg-His-Pro-Cys-His-Gly-Asn-Leu-Met-Asn-Gln-Ile-Lys

The amino acid sequences of several tryptic peptides were found to be:

1. His-Pro-Cys-His-Gly-Asn-Leu-Met-Asn-Gln-Ile-Lys
2. Met-Val-Ala-Tyr ...
3. Gly-Leu-Leu-Ser-Asn-Val-Leu-Cys ..
4. Leu-Gly-Cys-Gln-Leu-Leu-Gly-Thr-Tyr-Lys
5. Val-Leu-Asn-Pro-Thr-Ala-Val-Ser-Leu-Gln-Val-Lys
6. Asn-Gln-Leu-Ala-Gln-Leu-X-Gly-Ser-Ala-Asn-Ala-Leu-Phe-Leu-Val-Glu-Leu-Tyr ....
7. Leu-Val-Ile-Ser-Tyr...
8. Val-Gly-His-Val-Asp-Val-Pro-Val-Pro-Asp-His-Ser-Asp-Lys-Glu-Ala-Phe-Gln.
9. Leu-X-Ala-Thr-Ile-Asp-Val-Met-Arg.
10. Gln-Val-Ile-Ser-Val-Val-X-Gln-Ala-Phe.

The amino acid sequence of a peptide generated by digestion of LIF with the V8 protease was:

Ala-Phe-Gln-Arg-Lys-Lys-Leu-Gly-Cys-Gln-Leu-Leu-Gly-Thr-Tyr-Lys-Gln-Val-Ile-Ser-Val-Val-Val-Gln-Ala-Phe.

EXAMPLE 3

The following Example sets out the steps used to obtain radioactively-derivatized LIF and to identify sources of LIF by a receptor competition assay.

The accompanying Figure 6 relates to Example 3: Binding of radioiodinated-pure LIF (approximately 2 x

10^5 cpm/ng) to M1 myeloid leukaemia cells at 0°C. M1 cells (5 x 10^6) were incubated with ^{125}I-LIF (4 x 10^5 cpm) with or without competing factors for 2 hours after which bound radioactivity was separated from unbound radioactivity by centrifugation through foetal calf serum. A. Titration of unlabelled pure LIF and pure Multi-CSF, GM-CSF, G-CSF or M-CSF as competing factors at the indicated dilutions (10µl of each dilution added to a final volume of 80µl). The highest concentrations of each were 10^4 units/ml; 3.5 x 10^4 units/ml; 3 x 10^4 units/ml; 5 x 10^4 units/ml; 4 x 10^4 units/ml; respectively. B. Ability of pure LIF or lipopolysaccharide (LPS) or concentrated (4-8-fold) media conditioned by various cells or serum from endotoxin-injected mice to compete for the binding of ^{125}I-LIF to M1 cells as above.

LIF, purified from Krebs II ascites cell conditioned medium as described in Example 1, was radioactively labelled with ^{125}I on tyrosine residues as described previously (Nicola, N.A. and Metcalf, D., J.Cell.Physiol. 128:180-188, 1986) producing ^{125}I-LIF with a specific radioactivity of approximately 2 x 10^5 cpm/ng. ^{125}I-LIF bound specifically to M1 myeloid leukaemic cells as well as murine adult bone marrow, spleen, thymus and peritoneal exudate cells. Cell autoradiographic analysis indicated that ^{125}I-LIF bound specifically only to macrophages and their precursor cells, with receptor numbers increasing with cell maturation, and not detectably to any other haemopoietic cell type. This suggests that LIF might have clinically useful applications in modulating macrophage function in a variety of disease states. The specific binding of ^{125}I-LIF to M1 myeloid leukaemic cells or to peritoneal macrophage populations was inhibited by unlabelled LIF,

but not by a range of other haemopoietic growth factors, including G-CSF, GM-CSF, M-CSF (CSF-1), Multi-CSF (interleukin 3), interleukins 1,2,4,6, endotoxin or other growth factors (see for example Figure 6). The ability of cell conditioned media or cell extracts to inhibit the binding of ^{125}I-LIF to such cell populations (radioreceptor assay for LIF) (Figure 6B) thus provides a specific assay for the presence of LIF production or storage by various cells and tissues. For example, medium conditioned by lectin-activated LB3 T cells strongly inhibited specific binding of ^{125}I-LIF to M1 cells thus indicating that LB3 cells produce LIF.

EXAMPLE 4

The following Example sets out the steps used to obtain a cloned complementary DNA copy of the mRNA encoding murine LIF.

The accompanying Figures 7 to 10 relate to various steps of the method described below. In the Figures:

Figure 7 relates to Step 1 of the cloning of LIF cDNA clones: the accumulation of mRNAs for haemopoietic growth regulators in the cytoplasm of LB3 cells following stimulation with the lectin concanavalin A. Cytoplasmic polyadenylated RNA (5µg) from WEHI-3B D$^-$ cells (track 1), T cell clone LB3 cells cultured at 10^6 cells/ml with 5µg/ml concanavalin A for 5 hours (track 2), and unstimulated LB3 cells (track 3) was electrophoresed on formaldehyde agarose gels, transferred to nitrocellulose and hybridized with a ^{32}P-GM-CSF probe (panel a) or a ^{32}P-Multi-CSF probe (panel b) (for details see Kelso, A., Metcalf, D. and Gough,N.M. J.Immunol. 136:1718-1725, 1986).

Figure 8 relates to Step 3 of the cloning of LIF cDNA

clones: Synthetic oligonucleotide used for identifying LIF clones. A portion of the amino acid sequence of murine LIF near the N-terminus (residues 15-26, see Example 2) is shown on the top line, the possible combinations of mRNA sequences that could encode this peptide in the middle, and the oligonucleotide probe complementary to this mRNA sequence, below. Note that for all amino acid residues except Cys 17 and His 18, the oligonucleotide is complementary only to the codons most frequently used in mammalian coding regions (Grantham et.al., Nucleic Acids Res. 9:43-77, 1981). For Cys 17, the sequence is complementary to both codons. For His 18, the oligonucleotide is complementary to the less frequent CAU codon. It was considered unlikely that the CAC codon would be used as it would generate, in conjunction with the neighbouring Gly codon, the infrequent CpG dinucleotide (Swartz et.al, J. Biol. Chem. 238:1961-1967, 1962)

Figure 9 relates to Step 4 of the cloning of LIF cDNA clones; the identification of LIF cDNA clones by hybridization with the oligonucleotide illustrated in Figure 8.

Figure 10 relates to Step 5 of the cloning of LIF cDNA clones: nucleotide sequence of cDNA clone pLIF7.2b and LIF amino acid sequence. The sequence of the mRNA-synonymous strand of the cDNA is listed 5' to 3' with the predicted amino acid sequence of LIF given above; numbers at the end of lines indicate the position of the final residue (amino acid or nucleotide) on that line. The amino acid sequence is numbered consecutively from the first residue encoded in this clone. Regions of amino acid sequence determined by analysis of the protein are overlined; there are no discordancies between the two. Pro 9 was the N-terminal residue determined by direct

amino acid sequence analysis (Example 2). Seven potential N-linked glycosylation sites (Neuberger, A. et al in Glycoproteins, Gottschalk, A, ed., Elsevier, Amsterdam, pp 450-490, 1972) are indicated with asterisks and four potential O-linked glycosylation sites (Takahashi, N. et al, Proc. Natl. Acad. Sci. USA, 81: 2021-2025, 1984) with hatches.

Step 1: Preparation of RNA containing the LIF mRNA.

Cells of the cloned murine T cell line LB3 (Kelso, A. and Metcalf, D. Exptl.Hematol. 13:7-15, 1985) were stimulated with the lectin concanavalin A for 6 hours to enhance the accumulation in the cytoplasm of mRNAs encoding various factors which regulate the growth and differentiation of haemopoietic cells. (Kelso, A., Metcalf, D. and Gough, N.M. J.Immunol. 136:1718-1725, 1986). (For example, Figure 7 shows the increased production of mRNAs encoding the haemopoietic growth factors GM-CSF and Multi-CSF in cells so stimulated). Cytoplasmic RNA was prepared from 5 x 10^8 concanavalin A-stimulated LB3 cells using a technique described previously (Gough, N.M. J.Mol.Biol. 165:683-699, 1983). Polyadenylated mRNA molecules were partitioned from ribosomal RNA by two cycles of chromatography on oligo-dT cellulose using standard procedures (Maniatis, T. et.al., Molecular Cloning, Cold Spring Harbor, New York, 1982).

It was found subsequently by Northern blot analysis that unlike the mRNAs for GM-CSF and Multi-CSF, the LIF mRNA is present constitutively in LB3 cells and its abundance is not enhanced by concanavalin A stimulation (Gearing D.P. et al. EMBO J. 6: 3995-4002, 1987).

Step 2: Synthesis and cloning of a library of LB3 cDNA

molecules.

Double-stranded DNA copies of the LB3 mRNA prepared as described above were synthesized by standard procedures (Maniatis, T. et.al., Molecular Cloning, Cold Spring Harbor, New York, 1982 and Gough, N.M. et.al. Nature 309:763-767, 1984). Briefly, 10µg of cytoplasmic polyadenylated LB3 mRNA were used as a template for synthesis of single-stranded complementary DNA (cDNA) in a reaction catalyzed by avian myeloblastosis virus reverse transcriptase and primed with oligo-dT. After completion of this reaction, the mRNA was degraded by incubation at 65°C for 1 hour in 0.3 M NaOH, 1mM EDTA. After neutralisation of the base and recovery of the cDNA by ethanol precipitation, the single-stranded cDNA was converted to duplex form in a reaction catalyzed by the Klenow fragment of E.coli DNA polymerase I. The "hairpin loop" structure of the cDNA was then cleaved by treatment with the single-strand specific nuclease S1 and then tails of deoxycytidine residues (approximately 20-30 residues long) were appended to each end of the double-stranded cDNA using the enzyme terminal deoxynucleotidyltransferase as described (Michelson, A.M. and Orkin, S. J.Biol.Chem. 257:14773-14782, 1982). The dC-tailed cDNA was fractionated by electrophoresis on a 1.5% agarose gel and molecules greater than 500bp in length recovered and annealed to a plasmid DNA molecule (pJL3, Gough, N.M. et.al., EMBO J. 4:645:653, 1984) that had been cleaved with the restriction endonuclease Sac 1 and to which tails of deoxyguanosine residues had been appended (Michelson, A.M. and Orkin, J., J.Biol.Chem. 257:14773-14782, 1982). The tailed cDNA and plasmid molecules were annealed as described (Gough, N.M. et.al., Biochemistry 19:2702-2710, 1980) and E.coli MC1061 (Casadaban, M. and Cohen, S., J.Mol.Biol. 138:179-207, 1980) transformed with the

annealed cDNA/plasmid mixture and approximately 50,000 independently transformed bacterial colonies selected by growth on agar plates containing 10µg/ml ampicillin. The transformed bacterial colonies were removed from the agar plates by washing with liquid growth medium containing 50µg/ml ampicillin and stored as 10 independent pools in 10% glycerol at -70°.

Step 3: Design of oligonucleotide probes.

A sequence of 26 amino acids at the amino terminus of LIF and residues from 11 different peptides derived by digestion of LIF with trypsin and V8 protease have been determined (see Example 2). These amino acid sequences provided the basis for the design of oligonucleotides complementary to certain defined regions of the mRNA encoding LIF for use as hybridization probes to identify cDNA clones corresponding to the LIF mRNA.

Each naturally occurring amino acid is encoded in its corresponding mRNA by a specific combination of 3 ribonucleoside triphosphates (a codon) (eg Watson, J. Molecular Biology of the Gene, 3rd ed., Benjamin/Cummings, Menlo Park, Calif., 1976). Certain amino acids are specified by only one codon, whereas others are specified by as many as 6 different codons (eg Watson, J. Molecular biology of the Gene, supra). Since therefore a large number of different combinations of nucleotide sequences could in fact encode any particular amino acid sequence, a large number of different degenerate oligonucleotides would need to be constructed in order to cater for every possible sequence potentially encoding that peptide. There are however, certain technical constraints in the use of highly degenerate oligonucleotides as hybridization probes. However, since for a given amino acid not all codons are employed with equivalent frequency (Grantham,

R. et.al. Nucleic Acids Res. 9:43-73, 1981) and since in the mammalian genome the CpG dinucleotide is underrepresented, occurring at only 20-25% of the frequency expected from base composition (Swartz, M.N. et.al., J.Biol.Chem. 238:1961-1967, 1962), it is often possible to predict the likely nucleotide sequence encoding a given peptide and thus reduce the complexity of a given oligonucleotide probe. Given the foregoing considerations, a number of oligonucleotides corresponding to different LIF peptides were designed and synthesized by standard procedures.

Step 4: Screening of an LB3 cDNA library for LIF-encoding clones.

For screening colonies of bacteria by hybridization with oligonucleotide probes, 10,000-15,000 bacterial colonies from each pool of the aforementioned LB3 cDNA library were grown on agar plates (containing 50µg/ml ampicilin), transferred to nitrocellulose filter disks and plasmid DNA amplified by incubation of the filters on agar plates containing 200µg/ml chloramphenicol (Hanahan, D. and Meselson, M. Gene 10:63-67, 1980). After regrowth of colonies on the original plate, a second nitrocellulose filter was prepared as above. The master plate was regrown a second time and then stored at 4°C. Plasmid DNA was released from the bacterial colonies and fixed to the nitrocellulose filters as previously described (Maniatis, T. et.al. Molecular Cloning, Cold Spring Harbor, New York, 1982). Before hybridization, filters were incubated for several hours at 37°C in 0.9M NaCl, 0.09 M sodium citrate, 0.2% Ficoll., 0.2% polyvinyl-pyrollidone, 0.2% bovine serum albumin, 50µg/ml heat denatured salmon sperm DNA, 50µg/ml E.coli tRNA, 0.1M ATP and 2mM sodium

pyrophosphate. Hybridization was performed in the same solution, containing in addition 0.1% NP40, at 37°C for 18 hours. 500ng of the synthetic oligonucleotide shown in Figure 8, was radioactively labelled in a reaction catalyzed by polynucleotide kinase and containing 500 µCi of [γ-^{32}P]ATP (specific activity 2,000-3,000 Ci/mmol). Unincorporated [γ-^{32}P]ATP was then separated from the radioactively labelled oligonucleotide by ion exchange chromatography on a NACS-PREPAC column (Bethesda Research Laboratories) according to the manufacturer's instructions. The radioactively labelled oligonucleotide was included in the hybridization reaction at a concentration of approx.20ng/ml. After hybridization, filters were washed extensively in 0.9M NaCl, 0.09M sodium citrate, 0.1% sodium dodecyl sulphate at various temperatures (as described below) and after each wash, autoradiographed.

The rationale behind performing successive rounds of washing was that at lower temperatures, all clones having even a small degree of homology with the oligonucleotide (as little as approximately 15 nucleotides) would be revealed as autoradiographic spots appearing on duplicate filters. As the temperature is increased, the oligonucleotide probe would be melted from clones with the lowest level of homology and the corresponding autoradiographic signals would thus be lost. Clones with the highest degree of homology (and thus clones which represent the best candidates for containing LIF cDNA sequences) will retain hybridization at the highest temperature. By this strategy it is thus possible to focus directly on the strongest candidate clones. Figure 9 shows the performance of a set of clones on a duplicate pair of filters containing 15,000 LB3 cDNA clones as the washing temperature was raised from 46°C to

66°C. Several clones retained hybridization to the oligonucleotide only at lower temperatures whereas several retained hybridization even at 66°C (for example, clones 1 and 2). The latter clones were thus selected for further analysis and the corresponding bacterial colonies removed from the master plates, purified and plasmid DNA prepared from each bacterial clone by standard procedures (Maniatis, T. et.al. Molecular Cloning, Cold Spring Harbor, 1982). Preliminary structural analysis of these clones (by assessing the size of the inserted cDNA, by mapping the locations of cleavage sites for various restriction endonucleases and by testing for hybridization with various oligonucleotides corresponding to different LIF peptides) indicated that each of these clones were in fact identical; that is, they represented different isolates of the same original cloning event. Thus further detailed analysis was performed on only one clone, pLIF7.2b.

Step 5: Determination of the nucleotide sequence of pLIF7.2b.

Nucleotide sequence analysis of the cDNA portion of clone pLIF7.2b was performed by the dideoxy chain termination method (Sanger, F. et.al., Proc.Natl.Acad.Sci. USA 74:5463-5467, 1977), using alkaline or heat denatured double-stranded plasmid DNA as template, a variety of oligonucleotides complementary both to the regions of the vector (pJL3) flanking the cDNA and to sequences within the cDNA insert as primers, and using both the Klenow fragment of E.coli DNA polymerase I and the avian myeloblastosis virus reverse transcriptase as polymerases in the sequencing reactions. The sequence so determined for the cDNA portion of clone pLIF7.2b is shown in Figure 10. Such analysis confirmed that this clone does indeed

contain a DNA copy of a mRNA with the capacity to encode the LIF molecule, since within the only translational reading frame that spans the entire cDNA uninterrupted by stop codons, all of the amino acid sequences previously determined for various peptides of the LIF molecule may be found. This clone does not however contain a complete copy of the LIF coding region since (a) it does not extend at the 5' end through a region encoding a presumed hydrophobic leader sequence initiated by a methionine codon and (b) it does not include at its 3' end an in-frame translational stop codon. It does however extend at the 5' end past the start of the region encoding the mature protein, determined by comparison with the previously determined amino-terminal amino acid sequence (residue Pro 9 in Figure 10).

EXAMPLE 5

The following sets out the steps used to construct a full length copy of the murine LIF coding region, to install this coding region in a yeast expression vector and to produce murine LIF.

The accompanying Figures relate to various steps of the method described below. In the Figures:

Figure 11: relates to Step 1 of the expression of murine LIF in yeast cells: C-terminal amino acid sequence of LIF. The amino acid sequence at the C-terminus of the pLIF7.2b reading frame is shown above the sequence of a Staphylococcal V8 protease and a tryptic peptide derived from LIF purified from Krebs ascites tumour cells. Note that these latter peptides extend the pLIF7.2b LIF sequence by 9 amino acids and terminate at the same residue. The nucleotide and amino acid sequence at the corresponding region of clone pLIFNK1 is shown at the bottom line, confirming the C-terminus assigned by direct

amino acid sequencing. The numbering system is that of Figure 10.

Figure 12: relates to step 2 of the expression of murine LIF in yeast cells: reconstruction of the pLIF7.2b cDNA for insertion into the yeast expression vector, YEpsec1. Partial nucleotide sequence of the pLIF7.2b cDNA and its encoded amino acid sequence are shown on the top line. Numbering is according to Figures 10 and 11. The second and third lines show the sequence of pLIFmut1 and pLIFmut2 respectively. Asterisks indicate stop codons. Restriction sites (Bam HI, Hind III and Eco R1) used for cloning into YEpsec1 (Baldari. C. et al, EMBO J. 6: 229-234, 1987) and pGEX-2T (Smith, D.B. and Johnson, K.G., Gene, in press, 1988) are indicated. The bottom line shows partial sequence of the K.lactis killer toxin signal sequence in YEpsec1. The sequence Gly-Ser encoded at the Bam HI restriction site is efficiently recognized by signal peptidase (Baldari, C. et al, EMBO J. 6: 229-234, 1987).

Figure 13: relates to step 4 of the expression of murine LIF in yeast cells: the biological activity of yeast-derived recombinant LIF in cultures of M1 leukaemic cells. Titration in M1 cultures of yeast-derived recombinant LIF (-●-) versus purified native LIF-A (-o-) showing similar concentration dependent induction of differentiation in M1 colonies (panel A) and suppression of colony formation (panel B). Each point represents the mean value from duplicate cultures.

Figure 14: relates to step 5 of the expression of murine LIF in yeast cells: the ability of different dilutions of authentic native murine LIF-A (- o -), conditioned medium from yeast cells containing the LIFmut1/YEpsec1 recombinant induced with galactose (- ● -) and conditioned medium from the same yeast cells, but not induced with

galactose (- + -) to compete for the binding of native ^{125}I-LIF-A to cellular receptors on murine peritoneal cells.

Step 1: Determination of the amino acid sequence at the C-terminus of murine LIF.

The cDNA clone pLIF7.2b of Example 4 contains an incomplete copy of the murine LIF mRNA. At the 5' end, the cDNA encodes 8 residues N-terminal to the first residue determined by N-terminal amino acid sequence analysis (Pro 9 in Figure 10). At the 3' end however, pLIF7.2b is incomplete as it does not contain an in-frame translational stop codon. Inspection of the amino acid sequence of two LIF peptides determined by direct amino acid sequencing (Example 2) suggested that pLIF7.2b was lacking only 27 nucleotides of coding region at the 3' end. As illustrated in Figure 11, a V8-derived peptide started at residue Ala 162 of the cDNA sequence and extended the amino acid sequence deduced from the cDNA clone by 9 residues. A tryptic peptide contained within the V8 peptide terminated at the same residue, suggesting that Phe 187 is the C-terminal residue of the protein. In order to confirm this conclusion, a LIF cDNA clone overlapping with pLIF7.2b and extending 3'-ward was isolated and subjected to nucleotide sequence analysis.

In order to construct and screen an appropriate cDNA library from which to isolate such a clone, a series of different mRNA samples were screened by Northern blot hybridization to identify the RNA samples with the highest concentration of LIF mRNA molecules. Cytoplasmic polyadenylated RNA, prepared essentially as described previously (Gough, N.M. J.Mol.Biol. 165: 683-699, 1983), was fractionated on

1% agarose gels containing 20mM morpholinopropane sulfonic acid (MOPS), 5mM sodium acetate, 1mM EDTA (pH 7.0), plus 6% v/v formaldehyde, filters containing RNA were soaked in 2 x SSC, containing 0.2% Ficoll, 0.2% polyvinyl- pyrollidone, 0.2% bovine serum albumin, 2mM sodium pyrophosphate, 1mM ATP, 50µg/ml denatured salmon sperm DNA and 50µg/ml E.coli tRNA, at 67°C for several hours. Hybridization was in the same buffer plus 0.1% SDS at 67°C. The probe used to detect LIF transcripts consisted of an approx.750 bp Eco RI - Hind III fragment containing the cDNA insert of pLIF7.2b subcloned in pSP65. This fragment contains not only the cDNA sequence but also G-C tails and approx.150 bp of pJL3 vector sequence. Riboprobes of approx.2 x 10^9 cpm/µg were derived by transcription of this SP6 subclone using reagents supplied by BRESA (Adelaide). The probe was included in hybridization at approx.2 x 10^7 cpm/ml. Filters were washed extensively in 2 x SSC, 2mM EDTA, 0.1% SDS at 67°C and finally in 0.2 x SSC at 67°C prior to autoradiography.

Such analysis revealed that LIF transcripts were present at low levels in a wide variety of haemopoietic cell lines and that there was considerable variation in the level of LIF mRNA in different batches of Krebs RNA. Two batches of Krebs ascites tumour cell RNA were selected for synthesis of two cDNA libraries. cDNA libraries were constructed using the reagents supplied by Amersham (Product numbers RPN.1256 and RPN.1257) and using the manufacturer's instructions; the cloning vector was λGT10. Approximately 4 x 10^5 recombinant clones were obtained and screened by hybridization with an oligonucleotide corresponding to a sequence of 36 residues at the 3' end of the pLIF7.2b sequence: nucleotides 500-535 (inclusive) in Figure 10.

Phage plaques representing the Krebs cDNA library were grown at a density of approx.50,000 plaques per 10 cm petri dish, transferred in duplicate to nitrocellulose and treated using standard techniques (Maniatis, T. et al, Molecular Cloning, Cold Spring Harbor, 1982). Prior to hybridization, filters were incubated for several hours at 37°C in 6 x SSC (SSC = 0.15M NaCl, 0.015M sodium citrate), 0.2% Ficoll, 0.2% polyvinyl-pyrollidone, 0.2% bovine serum albumin, 2mM sodium pyrophosphate, 1mM ATP, 50 mg/ml denatured salmon sperm DNA and 50 µ/ml E.coli tRNA. Hybridization was in the same solution containing 0.1% NP40, at 37°C for 16-18 hours. The aforementioned oligonucleotide probe, radioactively labelled using [γ-^{32}P] ATP and polynucleotide kinase to a specific activity of approx.10^9 cpm/µg and separated from unincorporated label by ion exchange chromatography on a NACS column (Bethesda Research Laboratories), was included in the hybridization at a concentration of approx.20 ng/ml. After hybridization, filters were extensively washed in 6 x SSC, 0.1% sodium dodecyl sulphate at 60°C. One plaque representing clone λLIFNK1, positive on duplicate filters, was picked and rescreened at lower density, as before.

The cDNA insert of approx.950 bp in λLIFNK1, which hybridized with the aforementioned oligonucleotide, was subcloned into a plasmid vector (pEMBL8+, Dente, L. et al, Nucl.Acids Res. 11: 1645-1655, 1983) to generate clone pLIFNK1. Nucleotide sequence analysis of the cDNA insert in pLIFNK1 was performed by the dideoxy chain termination method (Sanger, F. et al, Proc.Natl.Acad.Sci. USA 74: 5463-5467, 1977) using alkaline denatured double-stranded plasmid DNA as template (Chen, E.Y. and Seeburg, P.H., DNA 4: 165-170, 1985), a variety of

oligonucleotides complementary both to regions of the vector flanking the cDNA and to sequences within the cDNA insert as primers, and using both the Klenow fragment of E.coli DNA polymerase and AMV reverse transcriptase as polymerases in the sequencing reactions. The nucleotide sequence of a portion of the cDNA insert in pLIFNK1 is shown in Figure 11. The amino acid sequence specified by pLIFNK1 is identical at the C-terminus with the 9 amino acids predicted by direct amino acid sequencing to constitute the C-terminus of LIF, and confirms that Phe 187 is the C-terminal residue of LIF, since in the pLIFNK1 cDNA sequence the codon for this residue is immediately followed by an in-frame translational stop codon.

Step 2: Construction of a LIF codon region in a yeast expression vector.

Initial production of the protein encoded by the LIF cDNA clones was achieved in a eukaryotic system (yeast), so that the expressed product would be glycosylated, secreted and correctly folded. The expression vector used, YEpsec1 (Baldari, C. et al, EMBO J. 6: 229-234, 1987), provides an N-terminal leader sequence derived from the killer toxin gene of Kluyveromyces lactis, shown previously to direct the efficient secretion of interleukin 1 (Baldari, C. et al, EMBO J. 6: 229-234, 1987), transcribed from a galactose-inducible hybrid GAL-CYC promoter.

In order to express the protein encoded by pLIF7.2b in this vector, it was necessary to modify the cDNA in several ways (see Figure 12). At the 5' end it was necessary not only to remove the few nucleotides specifying the partial mammalian leader sequence, but also to include an appropriate restriction

endonuclease cleavage site (Bam HI) to allow insertion in-frame with the K.lactis leader and retain an appropriate signal peptidase cleavage site (Gly-Ser). At the 3' end, two versions of the coding region for LIF were constructed. One version (LIFmut1) was constructed so that a stop codon immediately followed the last codon of pLIF7.2b (Gln 178). The other version (LIFmut2) was constructed to append a sequence encoding the 9 amino acid residues known to be missing from pLIF7.2b (see above), followed by an in-frame translational stop codon. An appropriate restriction endonuclease cleavage site (Hind III) completed both constructs. All of these modifications were achieved by oligonucleotide- mediated mutagenesis: The approx.750 bp Eco RI - Hind III fragment carrying the 535 bp cDNA insert of pLIF7.2b, bounded by G-C tails and a portion of the pJL3 vector was subcloned into plasmid pEMBL8+ (Dente, L. et al, Nucl.Acids Res. 11: 1645-1655, 1983) and single-stranded DNA was prepared as described (Cesarini, G. and Murray, J.A.H. in Setlow, J.K. and Hollaender, A. (eds), Genetic Engineering: Principles and Methods. Plenum Press, New York, Vol. 8, 1987 (in press)). In vitro mutagenesis was performed as described (Nisbet, I.T. and Beilharz, M.W. Gene Anal.Techn. 2: 23-29, 1985), using oligonucleotides of 35, 51 and 61 bases respectively, to modify the 5' end and the 3' end of the cDNA as outline above. Bam HI - Hind III fragments containing the modified LIF cDNA sequences were ligated into plasmid YEpsec1 and the nucleotide sequence of the inserts in the resulting recombinants was determined.

Step 3: Introduction of the YEpsec1/LIF recombinants into yeast cells.

S.cerevisiae strain GY41 (leu2 ura3 ade1 his4 met2 trp5 gal1 cir+; x 4003-5b from Yeast Genetic Stock Centre, Berkeley) was transformed by the polyethylene glycol method (Klebe, R.J. et al, Gene 25: 333-341, 1983). Transformants were selected and maintained on synthetic minimal medium (2% carbon source, 0.67% yeast nitrogen base (Difco) supplemented with 50µg/ml of the required amino acids) under uracil deprivation. Expression of insert sequences in plasmid YEpsec1 was achieved by growing transformants in either non-selective complete medium (1% yeast extract, 2% peptone) or in synthetic minimal medium, each containing 2% galactose.

Step 4: Determination of the biological properties of yeast-derived LIF.

Assays for differentiation-inducing activity and leukaemia-suppressive activity of yeast conditioned medium were performed in 1 ml cultures containing 300 M1 cells (provided by Dr. M. Hozumi, Saitama Cancer Research Centre, Japan) in Dulbecco's Modified Eagle's Medium with a final concentration of 20% foetal calf serum and 0.3% agar. Material to be assayed was added in serially diluted 0.1 ml volumes to the culture dish prior to the addition of the cell suspension in agar medium. Cultures were incubated for 7 days in a fully humidified atmosphere at 10% CO_2. Cultures were scored using a dissection microscope at x35 magnification, scoring as differentiated any colonies with a corona of dispersed cells or composed wholly of dispersed cells. Morphological examination of colonies was performed by fixing the entire culture with 1 ml 2.5% glutaraldehyde then staining the dried cultures on microscope slides using acetylcholinesterase/Luxol Fast

Blue/Haematoxylin.

Assays for colony stimulating activity were performed using 75,000 C57BL bone marrow cells as described previously (Metcalf, D. The Hemopoietic Colony Stimulating Factors Elsevier, Amsterdam, 1984). Assays for differentiation inducing activity on WEHI-3B D^+ cells were performed as described previously (Nicola, N. et al, J.Biol.Chem. 258: 9017-9023, 1983).

Medium from cultures of yeast containing the full-length coding region (LIFmut2), but not from cultures of non-transformed yeast, yeast containing the vector YEpsec1 alone, or yeast containing the incomplete coding region (LIFmut1), was able to induce typical macrophage differentiation in cultures of M1 colonies (Figure 13A). As with purified native LIF, with increasing concentrations the yeast-derived material also progressively reduced the number and size of M1 colonies developing (Figure 13B). Comparison with purified native LIF indicated that the yeast conditioned medium contained up to 16,000 Units/ml (approximately 130ng/ml) of LIF. Yeast-derived LIF, like purified native LIF-A, failed to stimulate colony formation by normal granulocyte-macrophage progenitor cells or to induce differentiation in, or suppress proliferation, of WEHI-3B D^+ leukaemic colonies.

Size fractionation on a Sephacryl S-200 column, indicated that yeast-derived LIF co-eluted with proteins of apparent molecular weight 67,000 to 150,000 Daltons, compared with 58,000 Daltons for LIF derived from Krebs cells.

The absence of LIF activity in medium conditioned by yeast containing an incomplete coding region (LIFmut1) suggests that the nine hydrophobic

C-terminal residues missing from this construct might be required for LIF function. Whilst these residues might interact with the receptor, they may also constitute part of a hydrophobic core within the protein, and thus their absence may prevent proper folding. Alternatively they might in some way be required for efficient secretion of LIF.

Step 5: Receptor binding specificity of yeast- derived murine LIF and purified native LIF-A from Krebs II ascites cell conditioned medium.

Purified murine LIF-A (Example 1) was iodinated as disclosed previously (Example 3). Peritoneal cells were harvested by lavage from mice in which a high level of macrophages had been induced in the peritoneal cavity with thioglycollate. These cells were washed and resuspended at 2.5 x 10^6/50µl in Hepes-buffered RPMI medium containing 10% foetal calf serum. Cells in 50µl aliquots were incubated with 200,000 cpm of ^{125}I-LIF-A (10µl, in the same medium) and 10µl of control medium or serial two-fold dilutions of unlabelled pure murine LIF-A or yeast-derived murine LIF. At appropriate concentrations, supernatant from galactose-induced yeast containing the LIFmut2 construct (Figure 12) competed for binding of ^{125}I-LIF-A to its receptors on peritoneal cells while uninduced yeast supernatant did not (Figure 14). The degree of competition was equal to that of authentic native LIF-A, indicating that the yeast-derived LIF-A contained all the information required for binding to LIF-A cellular receptors.

EXAMPLE 6.

The following example sets out the steps used to

express recombinant murine LIF in mammalian cells.

The accompanying Figure 15 relates to step 1 of the method described below: The nucleotide sequence of the cDNA portion of clone pLIFNK3. The nucleotide sequence of the mRNA-synonymous strand is presented in the 5' to 3' orientation with the inferred amino acid sequence of LIF given above. The N-terminal amino acid residue previously determined is indicated as +1. The Eco R1 site at the 5' end of the cDNA and the Xba I site spanning the stop codon (used in inserting the LIF coding region into pMP-Zen in step 2) are indicated.

Step 1: Determination of the amino acid sequence of the N-terminal leader sequence of murine LIF.

The cDNA clones pLIF7.2b and pLIFNK1 (see above) contain incomplete copies of the LIF mRNA. Although they contain, together, the complete coding region for the mature portion of the LIF protein, they do not contain the complete hydrophobic leader sequence, required for secretion of LIF from mammalian cells.

In order to isolate a cDNA clone containing the region encoding the hydrophobic leader, a further 10^6 cDNA clones constructed as above (Example 5), using the same batch of Krebs mRNA as a template, were screened (as above) using as probes two oligonucleotides corresponding to a sequence of 35 residues at the 5' end and 36 residues at the 3' end of the pLIF7.2b sequence (nucleotides 67-102 and 500-535, inclusive, in Figure 10).

Two plaques representing clones λLIFNK2 and λLIFNK3, positive on duplicate filters were picked and rescreened at lower density, as previously. Since λLIFNK3 was shown to hybridize with each of the two oligonucleotides used, it was selected for further analysis.

The cDNA insert of approx.1400bp in λLIFNK3 was subcloned into the plasmid vector pEMBL 8^+ (Dente, L., et.al., Nucleic Acids Res. 11:1645-1655, 1983) to generate clone pLIFNK3. Nucleotide sequence analysis of the cDNA insert of pLIFNK3 was performed as for pLIF7.2b and pLIFNK1 (above).

The nucleotide sequence of the cDNA insert in pLIFNK3 is shown in Figure 15, and indicates that pLIFNK3 contains a complete LIF coding region: there is an initiation codon (AUG) at position 23-25 in the pLIFNK3 sequence, preceding a sequence encoding a typical hydrophobic leader sequence of 24 amino acid residues. The coding region extends to the same translational stop codon as defined previously by pLIFNK1 (above).

Step 2: Introduction of a LIF coding region into a mammalian expression vector.

The mammalian expression vector chosen initially was the retroviral expression vector pMP-Zen. The vector is derived from the Moloney murine leukaemia virus-based vector pZIPNeo SV(X) (Cepko, C.L. et.al., Cell 37:1053-1062, 1984) by deletion of the neo^R gene together with nearby SV40 and plasmid sequences, leaving an Xho I expression site. The 3' region of the vector is also modified to incorporate the enhancer from the long terminal repeat (LTR) of the myeloproliferative sarcoma virus (MPSV) (Bowtel, D.D.L. et.al., Mol.Biol.Med. 4:229-250, 1987). This vector was chosen, firstly since the LIF/PMP-Zen recombinant may be packaged into helper-free infectious retroviral particles by passage through ψ2 cells (Mann, R., et.al. Cell, 33:153-159, 1983). Such viral particles may then be used to efficiently introduce (by infection) the LIF/pMP-Zen recombinant into a wide variety of murine cell types

(Mann, R. et.al. Cell 33:153-159, 1983). Secondly, this particular vector, which employs the MPSV LTRs for expression of the foreign coding region, has been shown to direct the efficient expression of certain other haemopoietic growth and differentiation factors including GM-CSF.

The segment of pLIFNK3 chosen for insertion into pMP-Zen extended from position 1 (an Eco RI site) to position 630 (an Xba 1 site spanning the stop codon) - see Figure 15. This segment was chosen since it contains little more than the coding region of pre-LIF and excludes all of the 3' untranslated region, which may contain sequences conferring mRNA instability (e.g. Shaw, G. and Kamen, R. Cell 46:659-667, 1986; Verma, I.M. and Sassone-Corsi, P., Cell 51 : 513-514, 1987). In order to insert this segment into pMP-Zen, it was first inserted between the Eco RI and Xba I sites of pIC20H (Marsh, J.L. et.al. Gene 32: 481-485, 1984) by standard techniques (Maniatis et.al., 1982, supra). The cDNA insert was then recovered from the polylinker of the pIC20H plasmid by Sal I plus Xho I digestion, thus generating a LIF cDNA fragment with cohesive ends appropriate for inserting into the Xho I cloning site of pMP-Zen. The insertion of the LIF cDNA fragment into pMP-Zen was achieved by standard techniques (Maniatis et.al., 1982, supra).

Step 3: Introduction of the pMP-Zen/LIF recombinant into murine fibroblasts.

pMP-Zen/LIF DNA was introduced into ψ2 fibroblasts by electroporation (Potter et.al., PNAS 81:7161-7165, 1984). 30μg pMP-Zen/LIF DNA plus 3μg pSV2Neo DNA (Southern, P.J. and Berg, P. J.Mol.App.Genet. 1:327-341, 1982) were mixed with 1 x 10^6 ψ2 fibroblasts in 1ml of DME/10% FCS (Dulbeccos modified Eagles medium containing 10% foetal calf serum) and

subjected to a pulse of 500v at a capacitance of 25μF (using a BioRad Gene-Pulser model No. 1652078). Transfectants were initially selected on the basis of resistance to the antibiotic G418 conferred by the pSV2Neo DNA. G418-resistant ψ2 cells were selected in 400μg/ml G418 by standard procedures (Mann, R. et al, Cell 33: 153-159, 1983). Of 19 G418-resistant clones examined, 2 also contained the pMP-Zen/LIF construct as assessed by LIF activity detectable in ψ2 conditioned media.

Step 4: Determination of the biological properties of ψ2-derived LIF.

Assays for differentiation-inducing activity and leukaemia suppressive activity of conditioned medium from pMP-Zen/LIF containing ψ2 cells were performed as described previously. Medium from cultures of 10^6 ψ2 cells in 3ml DME/10% FCS were assayed for differentiation-inducing activity. The results for two positive cultures are presented in the following table:

Clone No.	M1 Differentiation-inducing Activity (Units/10^6 cells/ml C.M.)
ψ2-control	not detectable
ψ2-11a	>16,000
ψ2-11d	>16,000

Thus, significant levels of biologically active, recombinant murine LIF can be produced in this expression system.

Step 5: Transmission of pMP-Zen/LIF retrovirus from ψ2 cells to haemopoietic cells.

Infectious pMP-Zen/LIF retrovirus could be transferred from the parent ψ2 cell lines to haemopoietic cells of the line FDC-P1 (Dexter, T.M. et.al., J.Exp.Med. 152:1036-1047, 1980) by cocultivation. 10^6 ψ2 cells were mixed with 10^6 FDC-P1 cells in 10ml DME/10% FCS containing the optimal concentration of WEHI-3BD$^-$ conditioned medium for growth of the FDC-P1 cells. Following 2 days incubation, the non-adherant FDC-P1 cells were removed, washed free from all adherant ψ2 cells and grown for 16 hours in 3ml of the same medium. Conditioned medium was harvested and assayed for differentiation-inducing and leukaemia suppressive activity as described previously. The results are shown in the following table:

FD Culture derived from Clone No.	M1 Differentiation-Inducing Activity (units/10^6 FDC-P1 cells)
ψ2-control	not detectable
ψ2-11a	approx. 3,000
ψ2-11d	approx. 1,500

Thus the ψ2 clones, ψ2-11a and ψ2-11d, are capable of transmitting biologically active pMP-Zen/LIF

retrovirus to haemopoietic cells. These clones should therefore be applicable to the infection of normal murine haemopoietic progenitor cells which can be used to reconstitute the haemopoietic system of irradiated mice in order to study the effects of high level expression of native LIF on normal murine haemopoiesis, in a manner analogous to that used for Zen/GM-CSF viral infection.

EXAMPLE 7.

The following sets out the steps used to express recombinant murine LIF in E.coli cells.

The accompanying Figure 16 relates to step 1 of the method described below: The nucleotide sequence at the glutathione S-transferase/thrombin cleavage site/LIF junction in plasmid pGEX-2T/LIF, and the sequence at the junction of the encoded fusion protein. The C-terminus of the glutathione S-transferase, the thrombin cleavage site and the N-terminus of the murine LIF portions of the tripartite fusion protein, along with the nucleotide sequence encoding this amino acid sequence, are shown. The expected site of cleavage by thrombin is indicated by an arrow.

Step 1: Introduction of a LIF coding region into an E.coli expression vector.

The expression vector used for expressing LIF in E.coli was pGEX-2T (Smith, D.B. and Johnson, K.S., Gene (in press), 1988), which directs the synthesis of foreign polypeptides as fusions with the C-terminus of Sj26, a 26kD glutathione S-transferase (E.C. 2.5.1.18) encoded by the parasitic helminth Schistosoma japonicum. In the majority of cases, fusion proteins have been shown to be soluble in aqueous solutions and can be purified from crude bacterial lysates under non-denaturing conditions by

affinity chromatography on immobilized glutathione. The particular vector pGEX-2T has been engineered so that the glutathione S-transferase carrier can be cleaved from the fusion protein by digestion with the site-specific protease thrombin.

The complete coding region of murine LIF, derived from plasmid pLIFmut2 (see Example 5 and Figure 12 above) was introduced as a Bam HI - EcoRI fragment into the multiple cloning site of pGEX-2T (Smith, D.B. and Johnson, K.S., supra), thus positioning the LIF coding region 3' of, and in the same translational reading frame as, that of the glutathione S-transferase and the thrombin cleavage site. Thus the LIF protein would be located C-terminal to these elements in a tripartite glutathione S-transferase/ thrombin cleavage site/LIF fusion protein (see Figure 16). Note that the position of the thrombin cleavage site is such that two amino acid residues (Gly-Ser) will be appended to the N-terminus of the LIF protein after thrombin cleavage. The construction of the aforementioned plasmid, pGEX-2T/LIF, was achieved by standard techniques, and the plasmid was introduced into E.coli NM522 (Gough, J.A. and Murray, N.M., J.Mol.Biol. 166: 1-19, 1983) by standard techniques.

Step 2: Expression and purification of glutathione S-transferase/LIF fusion protein.

In order to induce the expression of the glutathione S-transferase/LIF fusion protein, 10ml cultures of E.coli NM522 cells containing pGEX-2T/LIF were grown to logarithmic phase in Luria broth, and isopropyl β-D-thiogalactopyranoside added to a concentration of 0.1mM. After a further 4 hours growth, during which time the glutathione S-transferase/LIF gene is expressed, the cells were harvested by centrifugation and resuspended in

1ml mouse tonicity phosphate-buffered saline (MTPBS: 150mM NaCl, 16mM Na_2HPO_4, 4mM NaH_2PO_4, pH7.3). Cells were lysed on ice by mild sonication and after adding Triton X-100 to 1%, cell debris was removed by centrifugation (10,000g, 5 min, 4°C). The clarified supernatant was mixed at room temperature in a 50ml polypropylene tube on a rotating platform with 200µl 50% glutathione-agarose beads (sulphur linkage, Sigma). (Before use, beads were pre-swollen in MTPBS, washed twice in the same buffer and stored in MTPBS at 4°C as a 50% solution v/v). After absorption (5 min.), beads were collected by centrifugation (500g, 1 min) and washed three times in MTPBS/Triton X-100. The glutathione S-transferase/LIF fusion protein was then eluted by competition with free glutathione: beads were incubated with 100µl 50mM Tris. HCl, 5mM reduced glutathione (pH 7.5) for 2 min at room temperature and the beads removed by centrifugation. The elution step was performed twice, and the two 100µl aliquots of eluate pooled.

100µl of the glutathione S-transferase/LIF fusion protein was treated with thrombin as described (Smith, D.B. and Johnson, K.S., supra).

1µl aliquots of the uncleaved and thrombin-cleaved glutathione S-transferase LIF fusion proteins were electrophoresed on a Pharmacia Phast Gel (8-25% polyacrylamide gradient gel). After staining with Coomassie Blue, a single major protein species of relative molecular weight ~46 kDa was revealed in the uncleaved preparation, and in the thrombin-cleaved preparation two major bands of ~26 kDa and ~20 kDa (corresponding to the expected sizes of the fusion protein (46 kDa), the glutathione S-transferase (26 kDa) and the LIF proteins (20 kDa) respectively). By estimating the mass of the Coomassie-stained bands on this gel, the yield of

glutathione S-transferase/LIF protein from the original 10ml E.coli culture was estimated to be ~1.5µg.

Assays for differentiation-inducing activity and leukaemia-suppressive activity of both the glutathione S-transferase/LIF fusion protein and the thrombin cleaved LIF preparation, were performed using murine M1 cells (as described previously). Both preparations of LIF were determined to be biologically active, with specific activities in excess of $7x10^6$ units/mg.

EXAMPLE 8

The following sets out the steps used to determine whether the murine genome contains any other genes closely related to the gene encoding the sequence present in clone pLIF7.2b (Example 4) and the derivation of a map of the location of restriction endonuclease cleavage sites around the LIF gene.

The accompanying Figures relate to various steps of the method described below:

Figure 17: relates to step 1: hybridization of mouse DNA with a pLIF7.2b-derived probe under high and low stringency conditions. BALB/c liver DNA digested with the indicated restriction enzymes was probed for LIF gene sequences as described in Example 8, step 1. In the left hand panel, hybridization was at 65°C in 2 x SSC, and final washing at 65°C in 0.2 x SSC. In the right hand panel, hybridization and washing were both in 6 x SSC at 65°C. In the left hand panel, linear pLIF7.2b plasmid DNA (5.6 kb) was included at an amount equivalent to 10 and 1 copies per haploid mouse genome (400 pg and 40 pg respectively) assuming a molecular weight for the haploid mouse genome of 3×10^9 bp (Laird, C.D. Chromosoma, 32: 378-406, 1971). The sizes of the hybridizing genomic fragments are indicated.

Figure 18: relates to step 2: Hybridization of mouse DNA cleaved with various restriction endonucleases, singly and in pair-wise combinations, with a mouse LIF probe. Digested DNAs were electrophoresed on 0.8% agarose gels and hybridized with the pLIF7.2b cDNA fragment as described in Example 8, step 1, under high stringency conditions.

Figure 19: relates to step 2: Restriction endonuclease cleavage map of the murine LIF gene. The region of chromosomal DNA containing sequences corresponding to the cDNA clone pLIF7.2b is indicated by a box. The restriction sites given above (Eco R5) and below (Xba I, Bam HI, Pst I and Stu I) the central line have not been oriented with respect to the central line. The relative location of sites within the complex below the line is as indicated.

Figure 20: relates to step 3: chromosomal assignment of the murine LIF gene. In lane 1 BALB/c mouse embryo DNA was electrophoresed, in lane 2, DNA from Chinese hamster ovary cells and in lanes 3-8 DNA from the hybrid clones I-18A HAT, I-18A-2a- 8AG; EBS 58; EBS 11; EBS 4; and I-13A-1a-8AG (Cory, S. et al, EMBO J. 2: 213-216, 1983). The murine chromosome content of these hybrids is given in Cory, S. et al, (EMBO J. 2: 213-216, 1983). All DNAs were digested with Bam HI. The position of the 3 kbp Bam HI fragment bearing the murine LIF gene is indicated.

Step 1: Determination of the number of LIF-related genes in the murine genome.

In view of the data (Example 1) that medium conditioned by Krebs II cells contains two biochemically separable but functionally similar factors capable of inducing the differentiation of Ml cells (LIF-A and

LIF-B) we wished to determine how many genes there are in the murine genome related to the species we have purified and cloned. Southern blots of murine genomic DNA digested with various restriction endonucleases were therefore hybridized with a probe derived from pLIF7.2b under conditions of both high and low stringency.

20 µg aliquots of high molecular weight genomic DNA from BALB/c mouse livers were digested to completion with various restriction endonucleases, fractionated by electrophoresis in 0.8% agarose gels and transferred to nitrocellulose. Prior to hybridization, filters were incubated for several hours at 65°C in either 6 x SSC (low stringency) or 2 x SSC (high stringency) (SSC = 0.15M NaCl, 0.015M sodium citrate), 0.2% Ficoll, 0.2% polyvinyl-pyrollidone, 0.2% bovine serum albumin, 2mM sodium pyrophosphate, 1mM ATP, 50 µg/ml denatured salmon sperm DNA and 50 µg/ml E.coli tRNA. Hybridization was in the same solution containing 0.1% SDS, at 65°C for 16-18 hours. Filters were then washed in either 6 x SSC, 0.1% SDS at 65°C (low stringency) or in 2 x SSC, 0.1% SDS at 65°C, followed by 0.2 x SSC, 65°C (high stringency) as detailed in the legend to Figure 17. The hybridization probe used was as previously disclosed the approx.750 bp Eco RI - Hind III fragment spanning the cDNA insert of pLIF7.2b radiolabelled to a specific activity of approx.4 x 10^8 cpm/µg by nick-translation and included in the hybridization at a concentration of approx.2 x 10^7 cpm/ml.

In mouse liver DNA (Figure 17) as well as in DNA from Krebs II cells, LB3 T cells and WEHI265 monocytic cells (not shown) the LIF probe detected unique Eco RI, Bam HI, and Hind III fragments of approximately 11, 3 and 13 kbp respectively. The same pattern of hybridization

was evident at both high (0.2 x SSC, 65°C) and low stringency (6 x SSC, 65°C) (Figure 17); under low stringency hybridization and washing conditions no additional bands were evident. Similarly, unique hybridizing fragments were also detected in Pst I, Stu I, Sac I, Eco R5 and Bgl II-digested genomic DNA (Figure 17). Moreover, in the experiment illustrated in Figure 16, pLIF7.2b plasmid DNA was included at a concentration equivalent to 10 and 1 copies per haploid mouse genome (tracks 1 and 2); the hybridization intensity of the genomic LIF sequences was not significantly different from the intensity of the unique gene standard (track 2).

Taken together, the foregoing data indicate that the LIF gene is unique in the murine genome, with no close relatives. Thus the two species of LIF are not likely to be products of different genes, but rather are more likely to represent post- transcriptional or post-translational variants of the same gene product.

Step 2: Derivation of a restriction endonuclease cleavage map of the murine LIF gene.

In order to determine the disposition of restriction endonuclease cleavage sites in and around the murine LIF gene, and thus provide a molecular fingerprint of this gene, DNA from mouse livers or from Krebs ascites tumour cells was digested with various restriction endonucleases and subjected to Southern blot analysis as described in Step 1 (under high stringency hybridization and washing conditions). Examples of such experiments are shown in Figure 18. Analysis of the size of the digestion products enables one to generate a map of the location of each cleavage site around the LIF gene (Figure 19).

Step 3: Chromosome assignment of the murine LIF gene.

To determine the chromosome on which the mouse LIF gene is located, DNA was examined from six mouse-Chinese hamster ovary somatic cell hybrid cell lines which retain various mouse chromosomes (Cory, S. et al, EMBO J. 2: 213-216, 1983; Francke, U. et al, Cytogenet.Cell.Genet. 19: 57-84, 1977). Southern blot analysis performed as described in Step 1 (using high stringency hybridization and washing conditions) indicated that the 3 kbp Bam HI fragment containing the murine LIF gene was absent from all of the hybrids (Figure 20). Since chromosome 11 is the only mouse chromosome not retained in any of these lines (Cory, S. et al, EMBO J. 2: 213-216, 1983), a characteristic of mouse-Chinese hamster hybrids (Francke, U. et al, Cytogenet.Cell.Genet. 19: 57- 84, 1977), it is likely that the LIF gene is on this chromosome. On the same basis, the murine GM-CSF gene (Barlow, D.P. et al, EMBO J. 6: 617-623, 1987) and Multi-CSF gene (Ihle, J.N. and Kozak, C.A., National Cancer Institute, Frederick Cancer Research Facility, Annual Report, 1984) have also been assigned to chromosome 11, an assignment confirmed by genetic linkage studies (Barlow, D.P. et al, EMBO J. 6: 617-623, 1987).

EXAMPLE 9.

The following example sets out the steps used to establish conditions under which the cloned murine LIF cDNA can be used to identify by hybridization a human gene or mRNA or recombinant DNA clones containing human LIF-encoding sequences.

The accompanying Figure (Figure 21) relates to Step 2 of the method described below: hybridization of ^{32}P- labelled fragment of cDNA from clone pLIF7.2b under various conditions to genomic DNA of both mouse

and human origin. In each case, track 1 contains 15µg murine (LB3) DNA and track 2 and 3 contain 15µg human DNA (from the cell lines Raji or Ramos, respectively). The hybridization and washing conditions applied to each filter are described in Step 2. The approximately 10 kbp Eco RI fragment containing the murine LIF gene, and the approximately 9 kbp Eco RI fragment containing the human homologue, are arrowed. Molecular weight standards are given at the left. Two different autoradiographic exposures (16 hours and 62 hours) are shown.

Step 1: Preparation and Radioactive Labelling of a Fragment of cDNA from pLIF7.2b

pLIF7.2b plasmid DNA was amplified by growth in E.coli MC1061 (Casadaban, M. and Cohen, S., J.Mol.Biol. 138:179-207, 1980), extracted from the E.coli cells by standard procedures (Maniatis, J. et al, Molecular Cloning, Cold Spring Harbor, New York, 1982) and purified by CsCl-gradient centrifugations. pLIF7.2b plasmid DNA so purified was cleaved with the restriction endonucleases Eco RI and Hind III to release a cDNA-containing fragment of ~770 bp from the vector (pJL3), which was resolved from vector sequences by electrophoresis through a 1.5% agarose gel.

200 ng of pLIF7.2 cDNA fragment thus purified was radioactively labelled to a specific activity of approximately $3x10^8$ cpm/µg by nick-translation (Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P., J.Mol.Biol. 113:237-251, 1977) in a reaction containing 100µCi [α^{32}P]-dATP. The radioactively labelled cDNA fragment was purified from unincorporated label by precipitation in the presence of 1M sodium perchlorate and 33% isopropanol.

Step 2: Hybridization of Southern Blots of Mouse and Human Genomic DNA with a pLIF7.2b cDNA Probe

High molecular weight genomic DNA (15µg) from the murine T cell line LB3 (lane 1) and from two human B cell lines (Raji and Ramos; lanes 2 and 3) cleaved with the restriction endonuclease Eco RI was electrophoresed through a 0.8% agarose gel and transferred to nitrocellulose using standard techniques (Southern, E. M., J.Mol.Biol. 98:503-517, 1975). Five identical Southern blots were prepared containing each of these three DNAs. Before hybridization, filters were incubated for several hours at 55°C in either 0.9M NaCl, 0.09M sodium citrate, 0.2% Ficoll, 0.2% polyvinyl-pyrollidone, 0.2% bovine serum albumin, 50µg/ml heat denatured salmon sperm DNA, 50 µg/ml E.coli tRNA, 0.1mM ATP and 2mM sodium pyrophosphate (filters a, b, c, d) or in 0.3M NaCl, 0.03M sodium citrate, with the same additional components (filter e). Hybridization with the ^{32}P-labelled pLIF7.2b cDNA prepared in Step 1 was performed in the same solutions as the prehybridization, containing in addition 0.1% sodium dodecyl sulphate, at 55°C (filters a, b, c), or 65°C (filters d, e) for 16 hours. The ^{32}P-labelled cDNA was denatured by boiling prior to hybridization and was included in the hybridization reaction at ~10^7 cpm/ml.

After hybridization the filters were washed in either 0.9M NaCl, 0.09M sodium citrate, 0.1% sodium dodecyl sulphate at 55°C (filter a) 60°C (filter b) or 65°C (filters c, d) or in 0.3M NaCl, 0.03M sodium citrate, 0.1% sodium dodecyl sulphate at 65°C (filter e). After exhaustive washing, filters were auto-radiographed at -70°C using Kodak XAR-5 film and two intensifying screens. Figure 21 shows the results of such

an experiment, in which two of the hybridization/washing regimes described (filters d and e) allowed both the murine LIF gene (present on an approximately 10 kbp Eco RI fragment) and the human LIF gene (present on an approximately 9 kbp Eco RI fragment) to be detected above residual background non-specific hybridization. All other conditions tested gave rise to an unacceptably high level of background hybridization (filters, a, b, c).

EXAMPLE 10

The following example sets out the steps used to obtain a cloned human gene homologous to murine LIF.

The accompanying Figures relate to various steps of the method described below. In the Figures:

Figure 22: relates to step 1 of the cloning of the human LIF gene: Detection of the LIF gene by Southern blot hybridization using a mouse LIF cDNA probe. Genomic DNA from the human cell line RAMOS was digested with the indicated restriction endonucleases and hybridized under the conditions described in Example 19, step 1, with a mouse cDNA fragment derived from pLIF7.2b.

Figure 23: relates to step 1 of the cloning of the human LIF gene: detection of the LIF gene by Southern blot hybridization using a mouse LIF cDNA probe under a variety of hybridization conditions. Genomic DNA from the human cell line RAMOS (H) or mouse liver DNA (M) digested with the restriction endonuclease Bam HI were hybridized with a murine LIF cDNA probe (pLIF7.2b) under a variety of conditions of hybridization and washing. The temperatures and concentrations of SSC used for hybridization are indicated on the top line and the conditions of washing on the second line (see Example 10, step 1).

Figure 24: relates to step 2 of the cloning of the human

LIF gene: restriction endonuclease cleavage of three candidate LIF gene clones. 1 µg of DNA from λ phage of the clones λHGLIF1, λHGLIF2 and λHGLIF3 was digested with Sal I, Bam HI or Pst I, electrophoresed on an 0.8% agarose gel (left hand panel), transferred to nitrocellulose and hybridized (as disclosed previously) with pLIF7.2 cDNA. The approximate sizes of the hybridizing fragments are indicated.

Figure 25: relates to step 3 of the cloning of the human LIF gene: The nucleotide sequence of the mRNA- synonymous strand of a 1.3kbp segment of λHGLIF1 spanning the human LIE gene (H) is presented in a 5' to 3' orientation. The corresponding nucleotide sequence of the murine LIF mRNA (M) derived from cDNA clones pLIF7.2b, pLIFNK1 and pLIFNK3 is aligned beneath the human gene and given in lower case letters. Identities between the mouse and human sequences are indicated with asterisks. The presumed N-terminal residue of mature human LIF, by analogy with mouse LIF, is designated as +1.

Figure 26: relates to step 3 of the cloning of the human LIF gene: amino acid sequence of human LIF and comparison with mouse LIF. The amino acid sequence of mature murine LIE (M) as determined by direct amino acid sequencing, and analysis of the cDNA clones pLIF7.2b, pLIFNK1 and pLIFNK3 is listed on the top line, with the corresponding sequence of human LIF (H), as deduced from the sequence of λHGLIF1, listed below. Identities are indicated with asterisks.

Figure 27: relates to step 4 of the cloning of the human LIF gene: restriction endonuclease cleavage map of the human LIF gene. The exons of the human gene homologous with pLIF7.2b (Figure 25) are indicated as boxes. The direction of transcription of the gene is indicated by the arrow below the line.

Step 1: Detection of the human LIF gene with a mouse probe.

A method has been previously disclosed for using a radioactively labelled fragment of the mouse LIF cDNA clone pLIF7.2b as a hybridization probe to detect the human LIF gene. Figure 22 demonstrates that this method allows the human LIF gene to be detected in human genomic DNA digested with a variety of restriction endonucleases. Analyses such as this, and other gels not shown, revealed the sizes of DNA fragments generated by a variety of restriction endonucleases on which the human LIF gene is located. Such data is of importance in subsequent steps of this example, not only to establish conditions under which the mouse probe can be used as a hybridization probe to detect the human, but also to provide diagnostic restriction mapping data to aid in identifying human genomic LIF clones.

A high degree of homology between the mouse and human LIF sequences was apparent when mouse and human genomic DNA digested with Bam HI was hybridized with a mouse LIF cDNA probe under a variety of conditions of hybridization and washing (Figure 23). As the stringency of hybridization and washing was raised, so the background smear was reduced, revealing a unique fragment of ~3 kbp hybridizing with the mouse probe. Significantly, the human gene retained substantial hybridization even at 65°C in 0.2 x SSC.

Step 2: Screening of a human genomic library and isolation of a clone containing the LIF gene.

A library of human genomic DNA, partially digested with Sau 3A and ligated into the lambda phage cloning vector EMBL3A, was screened for LIF gene-

containing clones by hybridization with the mouse cDNA as a probe. The fragment of LIF cDNA was radioactively labelled and the conditions of hybridization were as disclosed previously (Example 4). Briefly, phage plaques representing the genomic library were grown at a density of ~50,000 plaques per 10 cm petri dish and transferred to nitrocellulose as described (Maniatis, T. et al, Molecular Cloning, Cold Spring Harbor, 1982). Prior to hybridization, filters were incubated for several hours at 65°C in 6 x SSC (SSC = 0.15M NaCl, 0.015M sodium citrate), 0.2% Ficoll, 0.2% polyvinyl-pyrollidone, 0.2% bovine serum albumin, 2mM sodium pyrophosphate, 1mM ATP, 50 µg/ml denatured salmon sperm DNA and 50 µg/ml E.coli tRNA. Hybridization as in the same solution containing 0.1% SDS, at 65°C for 16-18 hours. The LIF cDNA fragment, radioactively labelled by nick-translation using $[\alpha^{32P}]$ dATP to a specific activity of ~2 x 10^8 cpm/µg was included in the hybridization at a concentration of ~2 x 10^6 cpm/ml. After hybridization, filters were extensively washed in 6 x SSC, 0.1% sodium dodecyl sulphate at 65°C and then autoradiographed. Plaques positive on duplicate filters were picked and rescreened at lower density, as before.

Three clones were thus identified and purified: λHGLIF1, 2 and 3. In order to determine the relationship between these clones and to determine which, if any, contain the human LIF gene, DNA was prepared from each clone and digested with these restriction endonucleases: Sal I which liberates the entire segment of cloned genomic DNA and Bam HI and Pst I which cleave in and around the LIF gene to generate characteristic fragments of ~3 kbp and 1.8 kbp and 0.6 kbp respectively (as determined above). After digestion of

the recombinant phage DNAs and resolution by electrophoresis on agarose gels (Figure 24, left hand panel), the DNA was transferred to nitrocellulose and hybridized with the mouse LIF cDNA probe (under the conditions outlined above) to reveal the fragments containing the LIF gene (Figure 24, right hand panel). This analysis revealed that

(a) all three clones appeared to be identical;

(b) all contain an ~9 kbp segment of genomic DNA containing a region homologous with the mouse LIF probe;

(c) the region of homology with the mouse cDNA is present on a 3 kbp Bam HI fragment and on 1.8 and 0.6 kbp Pst I fragments, characteristics of the human LIF gene (see above). Thus it was concluded that all three clones contain a segment of chromosomal DNA encompassing the human LIF gene.

Step 3: Determination of the nucleotide sequence of the human LIF gene.

The ~3 kbp Bam HI fragment of λHGLIF1 shown above to hybridize with the mouse LIF cDNA probe was recloned into the plasmid vector pEMBL8$^+$, giving rise to clone pHGLIFBam1, and subjected to nucleotide sequence analysis. Nucleotide sequencing was performed by the dideoxy chain termination method (Sanger, F et al, Pro.Natl.Acad.Sci.USA. 74: 5463-5467, 1977) using alkaline denatured double-stranded plasmid DNA as template, a variety of oligonucleotides complementary to sequences within the gene as primers, and using both the Klenow fragment of E.coli DNA polymerase I and the avian myeloblastosis virus reverse transcriptase as polymerases in the sequencing reactions.

The entire nucleotide sequence of the Bam HI fragment spanning the LIF gene (2840bp) was determined, of

which 1297bp is shown in Figure 25. Alignment of this sequence with the murine LIF mRNA sequence revealed that the sequences encoding the mature human LIF protein are present on two exons separated by an intron of 693bp (Figure 25). For the region encoding the mature protein there is a high degree of homology between the two species at both the nucleotide and the amino acid sequence level. Within exon 1 there is 88% nucleic acid sequence homology (114/129 residues compared) and 91% amino acid sequence homology (39/43) for the region encoding the mature protein (position 58-186). Exon 2 is somewhat less homologous, 77% at the nucleotide level (318/411) and 74% at the amino acid level (101/136) within the coding region. Considering the mature protein as a whole, mouse and human LIF sequences determined here are identical at 140 of 179 positions (78%) with no insertions or deletions (Figure 26). Moreover, many of the differences are highly conservative substitutions (Lys:Arg, Glu:Asp and Leu:Val:Ala).

5' of the codon for the N-terminal proline residue of mature LIF, (position 58 in Figure 25) the human gene is homologous with the murine LIF mRNA sequence through a region encoding most of the hydrophobic leader. However, the entire leader is not encoded on this exon, since the mRNA and gene sequences diverge at a typical RNA splice site (TCCCCAG) (Mount, S.M., Nucleic Acids Res. 10:459-472, 1982). The exon specifying the 5' untranslated region and the first residues of the leader is not present within 1097 bp 5' of this splice site. In the mouse LIF gene, the exon specifying the first 6 amino acid residues of the hydrophobic leader is located ~1.5kbp 5' of the analogous splice site.

Step 4: Derivation of a restriction endonuclease cleavage

map of the human LIF gene.

In order to determine the disposition of the restriction endonuclease cleavage sites in and around the human LIF gene, and thus provide a molecular fingerprint of this gene, human genomic DNA (from the RAMOS cell line) was digested with various restriction endonucleases singly and in pair-wise combinations and subject to Southern blot analysis as described in Example 8, except that the probe used was the 3 kbp Bam HI fragment derived from pHGLIFBam1 described in Step 3 above and radio-labelled by nick-translation. Analysis of the data so-derived (not shown), as well as that shown in Figure 22 and derived from analysis of λHGLIF1 and pHGLIFBam1, gave rise to the restriction endonuclease cleavage map shown in Figure 27.

EXAMPLE 11

The following example details modifications made to the cloned human LIF gene in order to allow expression in yeast cells and determination of the biological and biochemical properties of the recombinant human LIF so derived.

In the Figures,

Figure 28: relates to step 1: oligonucleotides used to modify the human LIF gene for incorporation into YEpsec1. Oligonucleotide (a) corresponds to the 5' end of the coding region (residues 31 to 69 in Figure 25). Oligonucleotide (b) corresponds to the middle of the coding region (residues 163 to 186 and 880 to 903 in Figure 25). The portion of this oligonucleotide complementary to exon 1 is underlined with dashes, and that complementary to exon 2 with dots. Oligonucleotide (c) corresponds to the 3' end of the coding region (from position 1279 in Figure 25). Oligonucleotides (a) and (b)

introduce the indicated restriction endonuclease cleavage sites.

Figure 29: relates to step 1: nucleotide sequence of, and amino acid sequence encoded by, the synthetic human LIF cDNA derived by mutagene sis of the cloned human LIF gene. The Bam HI and Hind III cleavage sites introduced by oligonucleotides (a) and (c) (Figure 28) are indicated. The presumed N-terminal amino acid of mature LIF (by analogy with the mouse) is designated as +1.

Figure 30: relates to step 3: Induction of differentiation in colonies of M1 leukaemic cells by dilutions of purified native murine LIF (o------o) and conditioned medium from yeast cells containing the YEpsec1/HLIF recombinant induced with galactose (•____•). Medium from uninduced yeast cultures containing the YEpsec1/HLIF recombinant (o____o) was inactive. Mean data from replicate 7 day cultures is presented.

Figure 31: relates to step 4: competition of yeast-derived HLIF with native murine ^{125}I-LIF for binding to specific receptors on murine M1 cells. Dilutions of authentic native murine LIF-A (Example 1) (o____o), recombinant murine LIF (•____•) and conditioned medium from yeast cells containing the YEpsec1/HLIF construct uninduced (■____■) and induced with galactose (□____□) were tested for ability to compete for the binding of native ^{125}I-LIF-A to cellular receptors on murine M1 cells at 37°C, as disclosed previously.

Step 1: Modification of the human LIF gene for expression in yeast.

The isolation of a recombinant DNA clone containing the human LIF gene and the nucleotide sequence of the aforementioned gene has been previously disclosed.

(Example 10). The nucleotide sequence of 1297bp of DNA spanning the 2 exons encoding the mature human LIF protein is shown in Figure 25.

Murine recombinant LIF has previously been produced in yeast cells using the yeast expression vector, YEpsec1 (Example 5). This vector provides an N-terminal leader sequence derived from the killer toxin gene of Kluyveromyces lactis, transcribed from a galactose-inducible hybrid GAL-CYC promoter.

In order to express the protein encoded by the human LIF gene in this vector it was necessary to modify the gene in several ways. At the 5' end of the region encoding the mature protein a cleavage site for the restriction endonuclease Bam HI was introduced to allow insertion in frame with the K.lactis leader and retain an appropriate signal peptidase cleavage site (Gly-Ser). The same modification as previously applied to the mouse cDNA (pLIF7.2b) was made here. In the middle, the 693 bp intervening sequence was removed, fusing the two exons in the same translational reading frame. At the 3' end, a second translational stop codon was introduced immediately 3' of the natural stop codon, followed by a Hind III site for insertion into YEpsec1. All of the modifications were achieved by oligonucleotide-mediated mutagenesis: the ~3kbp Bam HI fragment spanning the LIF gene was subcloned into the plasmid pEMBL8$^+$, (Dente, L. et al, Nucleic Acids Res. 11:1645-1655, 1983) and single-stranded DNA prepared by F1 superinfection. In vitro mutagenesis was performed as described (Nisbet, I.T. and Beilharz, M.W. Gene Anal. Techn. 2:23 29, 1985), using oligonucleotides of 39, 48 and 39 bases respectively to modify the 5' end, the middle and 3' end of the gene as outlined above (see Figure 28). The nucleotide sequence of the modified human LIF coding region is given in Figure 29.

Step 2: Introduction of the YEpsec1/HLIF recombinant into yeast cells.

S.cerevisiae strain GY1$^+$ (leu2 ura3 ade2 trp1 cir+; from G. Cesareni, EMBL Heidelberg) was transformed by the polyethylene glycol method (Klebe, R.J. et al, Gene 25: 333-341, 1983). Transformants were selected and maintained on synthetic minimal medium (2% carbon source, 0.67% yeast nitrogen base (Difco) supplemented with 50µg/ml of the required amino acids) under uracil deprivation. Recombinant HLIF was produced by either of two methods. Either (1), Ura$^+$ transformants were grown to stationary phase in non-selective medium containing 2% galactose, and the medium assayed for LIF activity or (2), Ura$^+$ transformants were grown to stationary phase in selective minimal medium containing 2% glucose. Cells were then washed and resuspended in the same volume of selective minimal medium containing 2% ethanol and grown for 8 hours to overcome glucose repression. Transcription of the HLIF insert was then induced by diluting the cells (1:10) into synthetic minimal medium containing 2% galactose. Aliquots of culture supernatant were removed at various times after induction, filtered through Millipore filters (0.2µm) and assayed for LIF activity directly.

Step 3: Determination of the biological properties of yeast-derived HLIF.

In view of the high degree of sequence similarity between mouse and human LIF, the activity of yeast derived human LIF on murine M1 cells was assessed. Assays for differentiation-inducing activity and leukaemia-suppressive activity of yeast conditioned medium were performed in 1 ml cultures containing 300 murine M1 cells

(provided by Dr M. Hozumi, Saitama Cancer Research Centre, Japan) in Dulbecco's Modified Eagle's medium with a final concentration of 20% foetal calf serum and 0.3% agar. Material to be assayed was added in serially diluted 0.1 ml volumes to the culture dish prior to the addition of the cell suspension in agar medium. Cultures were incubated for 7 days in a fully humidified atmosphere of 10% CO_2 in air. Cultures were scored using a dissection microscope at x35 magnification, scoring as differentiated any colonies with a corona of dispersed cells or composed wholly of dispersed cells. Morphological examination of colonies was performed by fixing the entire culture with 1 ml 2.5% glutaraldehyde then staining the dried cultures on microscope slides using acetylcholinesterase/Luxol Fast Blue/Haematoxylin.

Medium from galactose-induced cultures of yeast containing the human coding region in YEpsec1, but not from uninduced cultures of the same yeast cells, from cultures of non-transformed yeast, or yeast containing the vector YEpsec1 alone, was able to induce typical macrophage differentiation in cultures of M1 colonies (Figure 30). As with murine LIF, with increasing concentrations the yeast-derived human material also progressively reduced the number and size of M1 colonies developing. Comparison with purified native murine LIF indicated that the yeast conditioned medium contained up to 50,000 Units/ml of human LIF.

Step 4: Receptor binding specificity of yeast-derived human LIF.

Purified murine LIF-A (Example 1) was iodinated as disclosed previously. M1 cells were washed and resuspended at 2.5 x 10^6/50µl in Hepes-buffered RPMI medium containing 10% foetal calf serum. Cells in 50 µl

aliquots were incubated with 200,000 cpm of ^{125}I-LIF-A (10µl in the same medium) and 10µl of control medium or serial two-fold dilutions of unlabelled pure murine LIF-A or conditioned medium from galactose-induced or uninduced cultures of yeast transformants containing the YEpsec1/HLIF construct.

Medium conditioned by galactose-induced yeast cells containing the YEpsec1/HLIF recombinant was able to compete for the binding of native murine ^{125}I-LIF-A to specific cellular receptors on murine M1 cells to the same extent as native and recombinant murine LIF-A, at 37°C. (Figure 31) and at 0°C (not shown). Medium from uninduced yeast cells and from yeast cells containing the vector YEpsec1 alone did not. Thus there appears to be a strong conservation of the receptor binding domain in murine and human LIFs, compatible with the high degree of primary amino acid sequence similarity.

Step 5: Purification, sequencing and iodination of yeast-derived human LIF.

Human LIF in medium conditioned by galactose-induced yeast cells containing the YEpsec 1/HLIF recombinant was purified using steps 2 and 4 of Example 1, except that the LIF activity binding to the DEAE-Sepharose CL-6B column and eluted with the salt gradient was pooled in step 2. Purified yeast-derived human LIF was radioiodinated by incubating 1µg of human LIF in 50µl of 0.2M sodium phosphate buffer, pH 7.2, with 1mCi of Na ^{125}I (2.7µl) and 5µl of 0.2mM ICl in 2M NaCl for 60 sec. ^{125}I-LIF was separated from unincorporated ^{125}I by passage of the reaction mixture through a column of Sephadex G-25M (Pharmacia) equilibrated in phosphate buffered (20mM, pH 7.4) saline (0.15M) containing 0.02% Tween 20 and 0.02% sodium azide. Human ^{125}I-LIF

electrophoresed on a 8-25% gradient sodium dodecyl sulphate polyacrylamide gel as a single broad band with apparent molecular weight of 170,000. Human ^{125}I-LIF bound specifically to murine M1 cells and bone marrow cells confirming the ability of human LIF to bind to murine LIF receptors. Purified yeast-derived human LIF (approx. 10µg) was subjected to amino-terminal amino acid sequencing as described in Example 2 and gave a single sequence of:

Ile-Thr-Pro-Val-X-Ala ..... .

This is identical to the predicted amino acid sequence of human LIF (Figure 26) except that the sequence begins at the fourth amino acid compared to the start of the murine sequence (see Example 2) which is:

Pro-Leu-Pro-Ile-Thr-Pro-Val-Asn-Ala ..... .

This indicates that the purified yeast-derived human LIF is missing the corresponding first three amino acids of the mouse sequence but is still biologically active and still able to bind to the murine LIF receptor. The first three amino acids thus appear to be dispensable for the biological activity of LIF.

EXAMPLE 12

The following example sets out the steps used to identify and partially purify a putative native human LIF molecule.

The accompanying Figures relate to various steps of the method described below. In the Figures:

Figure 32: relates to step 2 of the identification of a putative human LIF: the ability of different dilutions of medium conditioned by the human bladder carcinoma cell line 5637 (ATCC No. HTB9) (- □ -) crude or (- + -) DEAE non-binding fraction or of native murine LIF-A (- o -) to compete for the binding of murine ^{125}I-LIF-A to

cellular receptors on murine peritoneal cells. The inability of human G-CSF (- □ -) to compete for binding is also shown (undiluted = 5µg/ml).

Figure 33: relates to step 3 of the identification of a putative human LIF: the fractionation of medium conditioned by the human bladder carcinoma cell line 5637 on a column of DEAE- Sepharose CL-6B, eluted exactly as for the previously described fractionation of murine LIF-A (Example 1), and the ability of individual fractions from this fractionation to induce the formation of differentiated colonies of murine Ml cells. Panel A shows the salt gradient, Panel B shows the fractionation of 5637 conditioned medium; Panel C shows the fractionation of Krebs II cell conditioned medium for comparison.

Figure 34: relates to step 3 of the identification of a putative human LIF: the fractionation of medium conditioned by the human bladder carcinoma cell line 5637 on a column of lentil-lectin Sepharose 4B eluted as previously described for the fractionation of murine LIF-A (Example 1) and the ability of individual fractions from this fractionation to induce the formation of differentiated colonies of murine Ml cells. Panel A shows the gradient of α-methyl-D-mannopyrannoside, Panel B shows the fractionation of 5637 conditioned medium and Panel C shows the fractionation of Krebs II conditioned medium for comparison.

Step 1: Medium conditioned by several human cell lines were assayed for their capacity to induce the differentiation and inhibit the proliferation of Ml murine myeloid leukaemic cells in semi-solid agar cultures (as described in Example 5, Step 4 of the present application). Several cell lines produced such an

activity, of which the bladder carcinoma cell line 5637 produced the highest levels. However, the 5637 cell line has been shown previously to produce human G-CSF (Nicola, N.A. et al, Nature 314: 625-628, 1985; Welte, K. et al, Proc.Natl.Acad.Sci. USA 82: 1526-1530, 1985) which is also active in inducing differentiation of Ml cells (Tomida, M. et al, FEBS Lett. 207: 271-275, 1986; Neckers, L.M. and Pluznik, D.H. Exp.Hematol. 15: 700-703, 1987). To confirm that 5637 cells produced authentic human LIF (in addition to G-CSF), 5637-conditioned medium was further subjected to Steps 2 and 3 below.

Step 2: Medium conditioned by 5637 cells was concentrated 20- fold and tested for its ability to compete for the binding of native murine ^{125}I-LIF-A to specific cellular receptors on murine peritoneal cells. This competition binding assay was as disclosed in Step 5 of Example 5 in the present application. 5637 cell-conditioned medium contained activity capable of competing for the binding of murine ^{125}I-LIF-A to cellular receptors and this activity was concentrated in the DEAE non-binding fraction of 5637 CM (LIF-A) (Figure 32). Since human and murine G-CSF do not compete, even at very high concentrations, for ^{125}I-LIF-A binding sites, this establishes the presence in 5637-conditioned medium of a homologous human LIF activity capable of recognizing specifically the murine LIF receptor. This indicates strong conservation of the murine and human LIFs in their receptor binding domain, compatible with the high degree of primary amino acid sequence homology.

Step 3: Medium conditioned by human bladder carcinoma 5637 cells (two litres in 10% v/v foetal calf serum) was

concentrated to 40 ml and chromatographed sequentially on DEAE-Sepharose CL-6B and lentil-lectin-Sepharose 4B as described previously for murine LIF from Krebs II ascites cell-conditioned medium. M1 differentiation-inducing activity from 5637 cells (putative human LIF) chromatographed in a very similar fashion to murine LIF on DEAE-Sepharose, with some activity not binding to the column (LIF-A), while the remainder bound and was eluted during the salt gradient (LIF-B) (Figure 33). Similarly, the putative human LIF behaved like murine LIF on lentil-lectin-Sepharose chromatography with a proportion of activity binding to the column indicating the presence of mannose-containing carbohydrates on the glycoprotein (Figure 34). Thus by its cross-reactivity in inducing murine M1 cell differentiation, its ability to recognize specifically the murine LIF cellular receptor, and its biochemical fractionation characteristics, the human activity in 5637 cell conditioned media meets the criteria of the native human analogue of murine LIF.

Native human LIF from 5637 cell conditioned medium was purified by steps 2 and 4 of Example 1, pooling the non-binding LIF activity from step 2 and the binding LIF activity from step 4. This pooled LIF activity was fractionated by reverse-phase HPLC as for step 5 of Example 1 except that a Brownlee RP300 C8 column was used twice, first using a gradient from 0-60% CH_3CN in 0.1% TFA and then using a gradient from 45-55% CH_3CN in 0.1% TFA. In the second gradient human LIF eluted at 50% CH_3CN and when electrophoresed on 8-25% gradient sodium dodecyl sulphate polyacrylamide gels showed a major silver staining band of apparent molecular weight 73,000. Native purified human LIF was radioiodinated as described in step 5 of Example 10 and bound specifically to murine M1 cells and mouse bone marrow cells as described for yeast-derived human ^{125}I-LIF (step 5 of Example 11).

CLAIMS:

1. A composition comprising leukemia-inhibitory factor (LIF) in essentially pure form.

2. The composition of claim 1 wherein the LIF is murine LIF.

3. The composition of claim 1 wherein the LIF is human LIF.

4. A method of purifying glycosylated or non-glycosylated LIF, which comprises (a) chromatographing LIF in crude form on an anion exchange column, and eluting LIF by an increasing salt gradient, (b) chromatographing the product of step (a) on a lectin affinity column, said lectin having an affinity for mannose, and eluting LIF with a mannose derivative, (c) chromatographing the product of step (b) on a cation exchange column and eluting LIF with an increasing salt gradient, (d) chromatographing the product of step (c) under HPLC conditions on a reverse phase column and eluting LIF with an increasing gradient of acetonitrile.

5. The method of claim 4, wherein the LIF in crude form comprises a Krebs II ascited tumour cell conditioned medium, and murine LIF is purified from said medium.

6. The method of claim 4, wherein the LIF in crude form comprises human bladder carcinoma cell line 5637 conditioned medium, and human LIF is purified from said medium.

7. A recombinant DNA molecule comprising a nucleotide sequence which codes on expression in a suitable host cell for a polypeptide having LIF activity.

8. A recombinant DNA molecule comprising a nucleotide sequence which codes for a polypeptide having the amino acid sequence selected from the group consisting of those set forth in Figures 15, 26 and 29.

9. The molecule of claim 7, wherein said nucleotide sequence hybridizes to a nucleic acid probe corresponding to a subsequence of a LIF encoding insert selected from the group consisting of the LIF encoding inserts of clones pLIF7.2b, pLIFNK2, pLIFNK3, and pHGLIFBam1.

10. The molecule of claim 6, wherein the nucleotide sequence codes on expression in a eukaryotic host for a polypeptide which comprises a mature polypeptide which is at least partially homologous with the mature polypeptide amino acid sequence selected from the group consisting of those set forth in Figures 15, 26 and 29.

11. The molecule of claim 10, wherein the mature polypeptide is fully homologous with the amino acid sequence set forth in Figure 29.

12. The molecule of claim 7, wherein said nucleotide codes on expression for a polypeptide which is bound by cellular receptors which bind LIF, said binding being competitively inhibited by native murine or human LIF.

13. The molecule of claim 7, further comprises an origin of replication, whereby said molecule is rendered suitable for use as a cloning vector.

14. The molecule of claim 13, further comprising a promoter sequence operably linked to said nucleotide sequence whereby said molecule is rendered suitable for use of an expression vector.

15. A host cell transformed with a recombinant DNA molecule according to claim 14, wherein said cell expression a polypeptide having LIF actually under the control of said promoter.

16. A method of producing a polypeptide having LIF activity which comprises: (a) providing host cells according to claim 13, (b) cultivating said cells under conditions conducive to expression of a polypeptide having LIF activity; and (c) recovering said polypeptide.

17. The method of claim 16, wherein the polypeptide comprises a mature polypeptide which is at least partially homologous with the amino acid sequence selected from group consisting of those set forth in Figures 15, 26 and 29.

18. The method of claim 16, wherein the mature polypeptide is fully homologous with amino acid sequence set forth in Figure 29.

19. The method of claim 16, wherein the polypeptide is glycosylated by the host cell.

20. The method of claim 16, wherein the host cell is a yeast cell.

21. The method of claim 16, wherein the promoter is a galactose inducible hybrid GAL-CYC promoter.

22. The method of claim 16, wherein said molecule further comprises a signal sequence operably linked to said nucleotide sequence, said signal sequence coding an expression for a leader sequence directing the secretion of the polypeptide.

23. The method of claim 22, wherein the signal sequence is derived from the signal sequence of the killer toxin gene of Kluyveromyces lactis.

24. The method of claim 20, in which the host cell is of the species Saccharomyces cerevisiae.

25. The method of claim 16, wherein the host cell is a mammalian cell.

26. The method of claim 25, wherein the host cell is transformed with a retroviral expression vector.

27. The method of claim 25, wherein the vector is derived from the Moloney murine leukemia virus.

28. The method of claim 27, wherein the vector further comprises the LTR enhancer of myeloproliferative sarcoma virus.

29. The method of claim 28, wherein said vector lacks the 3' untranslated region of native LIF mRNA.

30. The method of claim 25, wherein the host cells are hemopoietic cells.

31. The method of claim 16, wherein the host cells are E.coli cells.

32. The method of claim 31, wherein the vector is one which directs the expression of a glutathione S-transferase/LIF fusion protein.

33. The method of claim 32, wherein the vector is one which directs the expression of a glutathione S-transferase/thrombin cleavage site/LIF fusion protein.

34 A polypeptide having LIF activity, prepared by the method of claim 16.

35. A polypeptide having LIF activity which is not identical in amino acid sequence to any naturally occurring form of LIF.

36. The polypeptide of claim 35, which has an amino acid sequence comprising amino acid residues 179-187 of natural murine LIF.

37. The polypeptide of claim 36, which has an N-terminal which lacks at least the first three amino acid residues of natural murine LIF.

38. A method of hindering the proliferation of myeloid leukaemia cells which comprises administering a pharmaceutically effective dose of polypeptide having LIF activity in a pharmaceutically acceptable form.

39. The method of claim 38, which further comprises administering a polypeptide having G-CSF or GM-CSF activity.

40. A pharmaceutical composition comprising a polypeptide having LIF activity in association with one or more pharmaceutically acceptable carriers and/or diluents.

41. The composition of claim 40, wherein said polypeptide comprises a mature polypeptide which is at least partially homologous with the amino acid sequence selected from the group consisting of those set forth in Figures 15, 26 and 29.

42. The composition of claim 41, wherein the mature peptide is fully homologous with the amino acid sequence set forth in Figure 29.

43. The composition of claim 40, further comprising at least one other biological regulator of blood cells.

44. The composition of claim 43, wherein said other regulator is a polypeptide having G-CSF or GM-CSF activity.

45. A diagnostic reagent or probe comprising at least one nucleic acid sequence corresponding to a sequence or subsequence of an LIF encoding insert selected from the group consisting of the LIF-encoding inserts of clones pLIF7,2b, pLIFNK1, pLIFNK3, and pHGLIFBam1, or to a sequence or subsequence of the corresponding mRNAs.

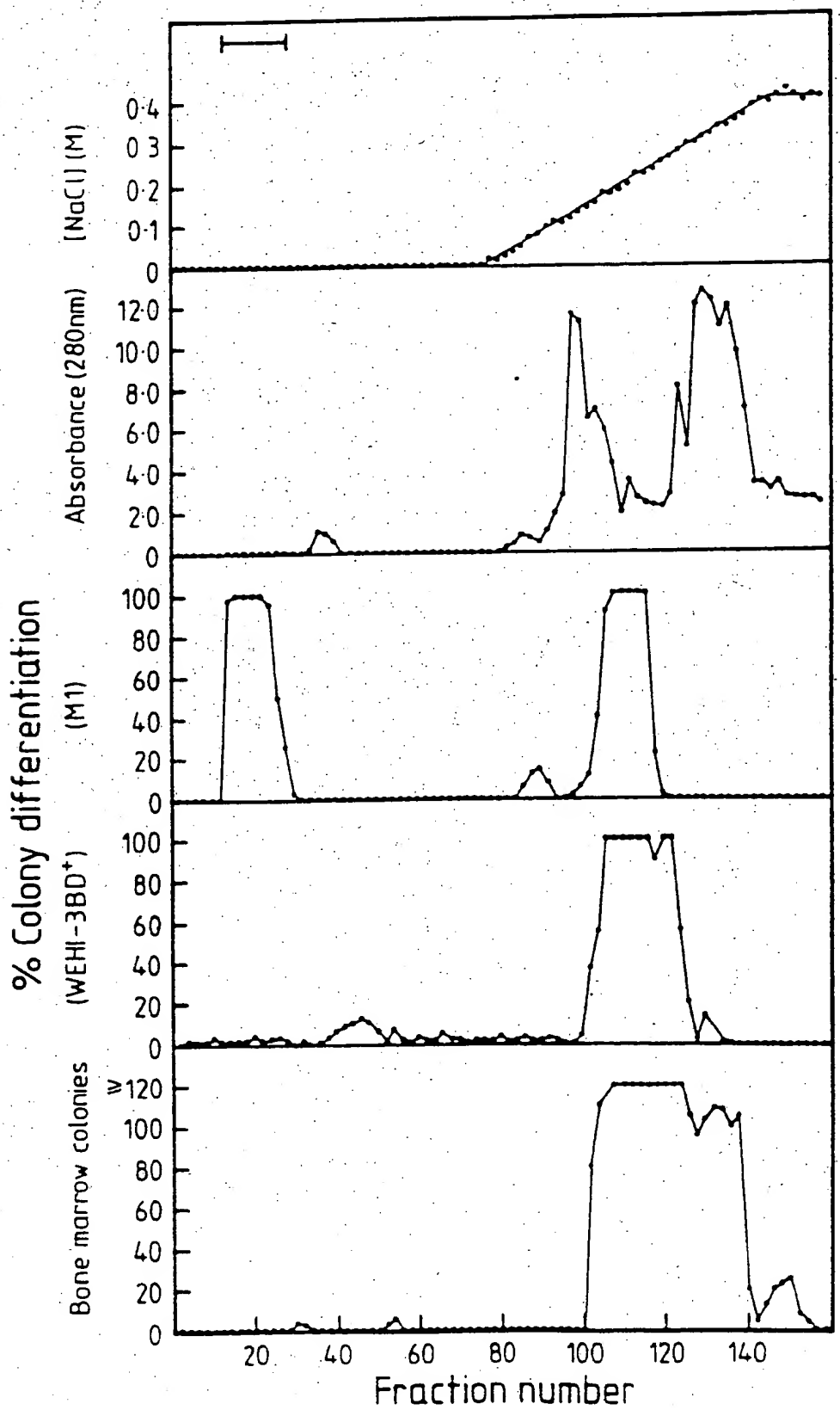

[NaCl] (M)
0·4
0·3
0·2
0·1
0
Absorbance (280nm)
12·0
10·0
8·0
6·0
4·0
2·0
0
% Colony differentiation
(M1)
(WEHI-3BD+)
100
80
60
40
20
0
Bone marrow colonies
≥120
20
40
60
80
100
120
140
Fraction number

FIG. 1.

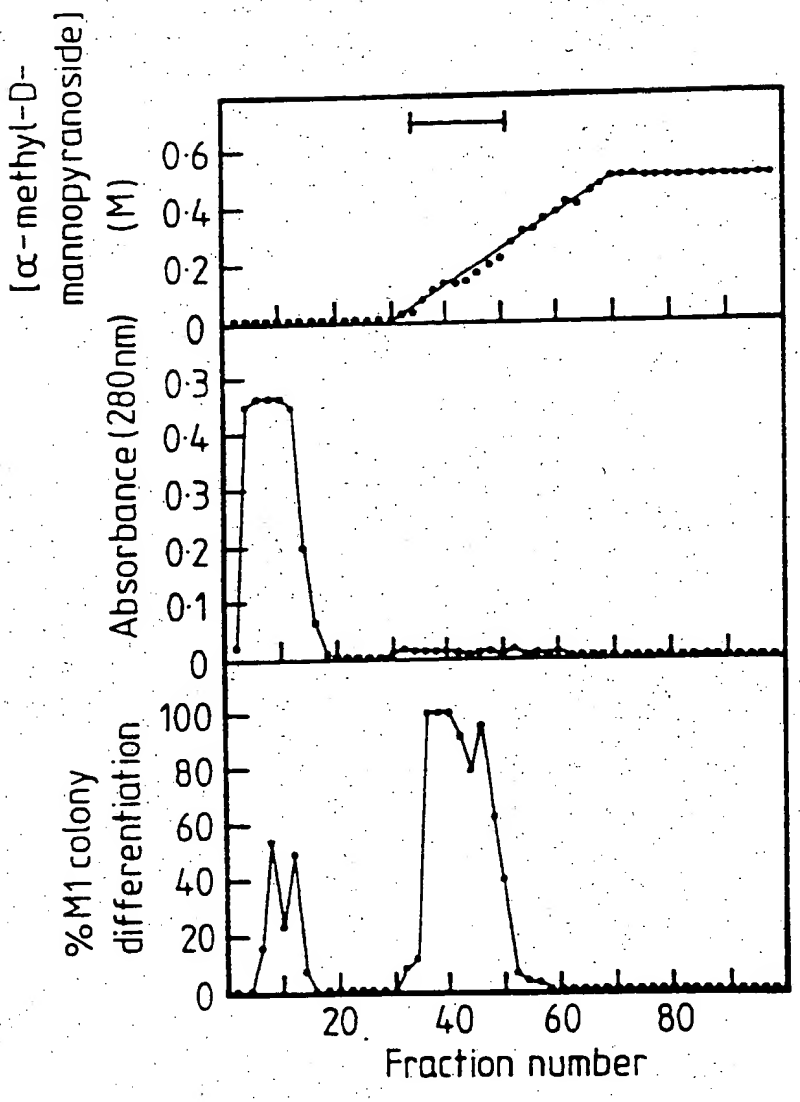

[α-methyl-D-mannopyranoside] (M)
0·6
0·4
0·2
0
Absorbance (280nm)
0·3
0·4
0·3
0·2
0·1
0
% M1 colony differentiation
100
80
60
40
20
0
20
40
60
80
Fraction number

FIG. 2.

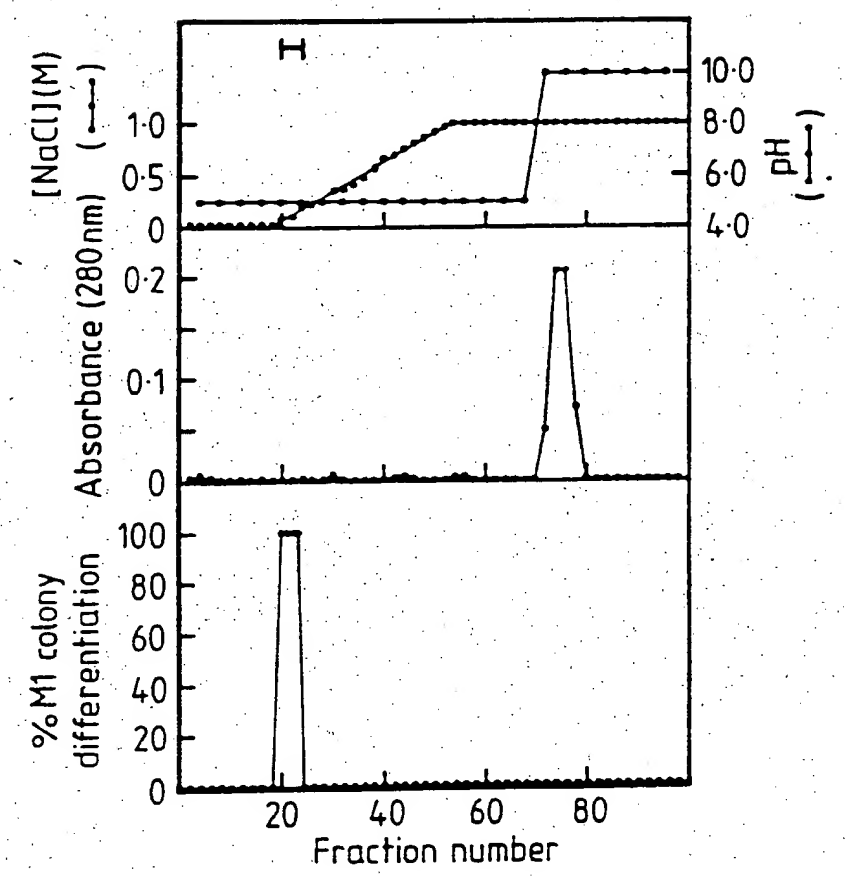

H
[NaCl](M)
1·0
0·5
0
Absorbance (280nm)
0·2
0·1
0
% M1 colony differentiation
100
80
60
40
20
0
20
40
60
80
Fraction number
10·0
8·0
6·0
4·0
pH

FIG. 3.

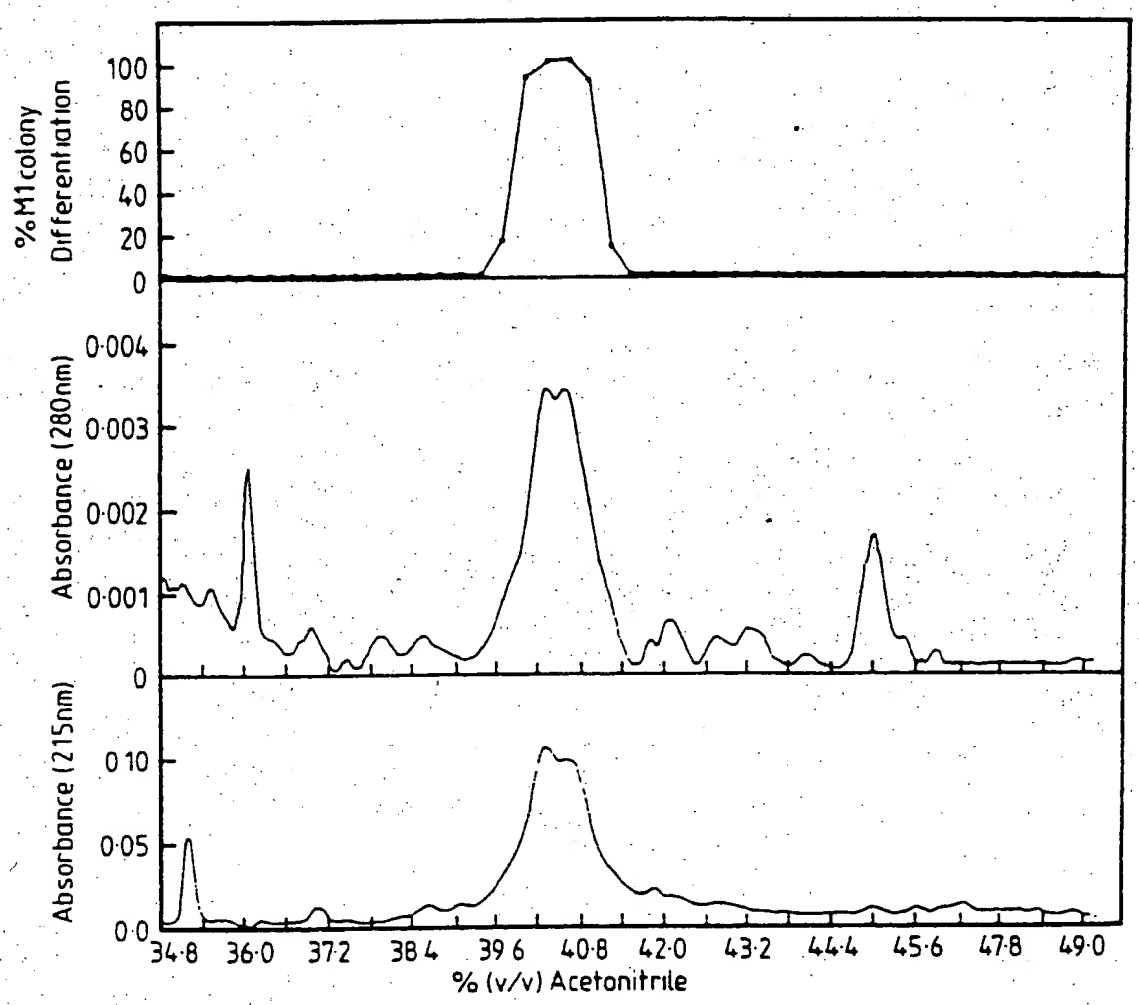

% M1 colony Differentiation
100
80
60
40
20
0
Absorbance (280nm)
0·004
0·003
0·002
0·001
0
Absorbance (215nm)
0·10
0·05
0·0
34·8
36·0
37·2
38·4
39·6
40·8
42·0
43·2
44·4
45·6
47·8
49·0
% (v/v) Acetonitrile

FIG. 4.

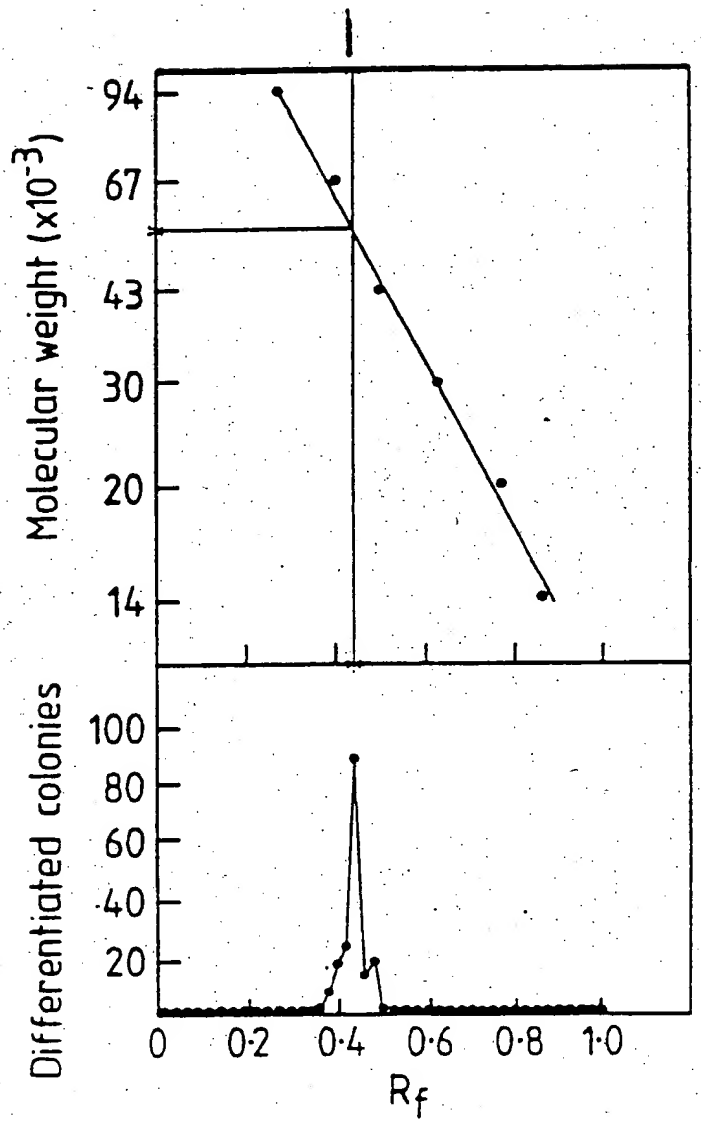

Molecular weight (x10^{-3})
94
67
43
30
20
14
Differentiated colonies
100
80
60
40
20
0
0·2
0·4
0·6
0·8
1·0
R_f

FIG. 5.

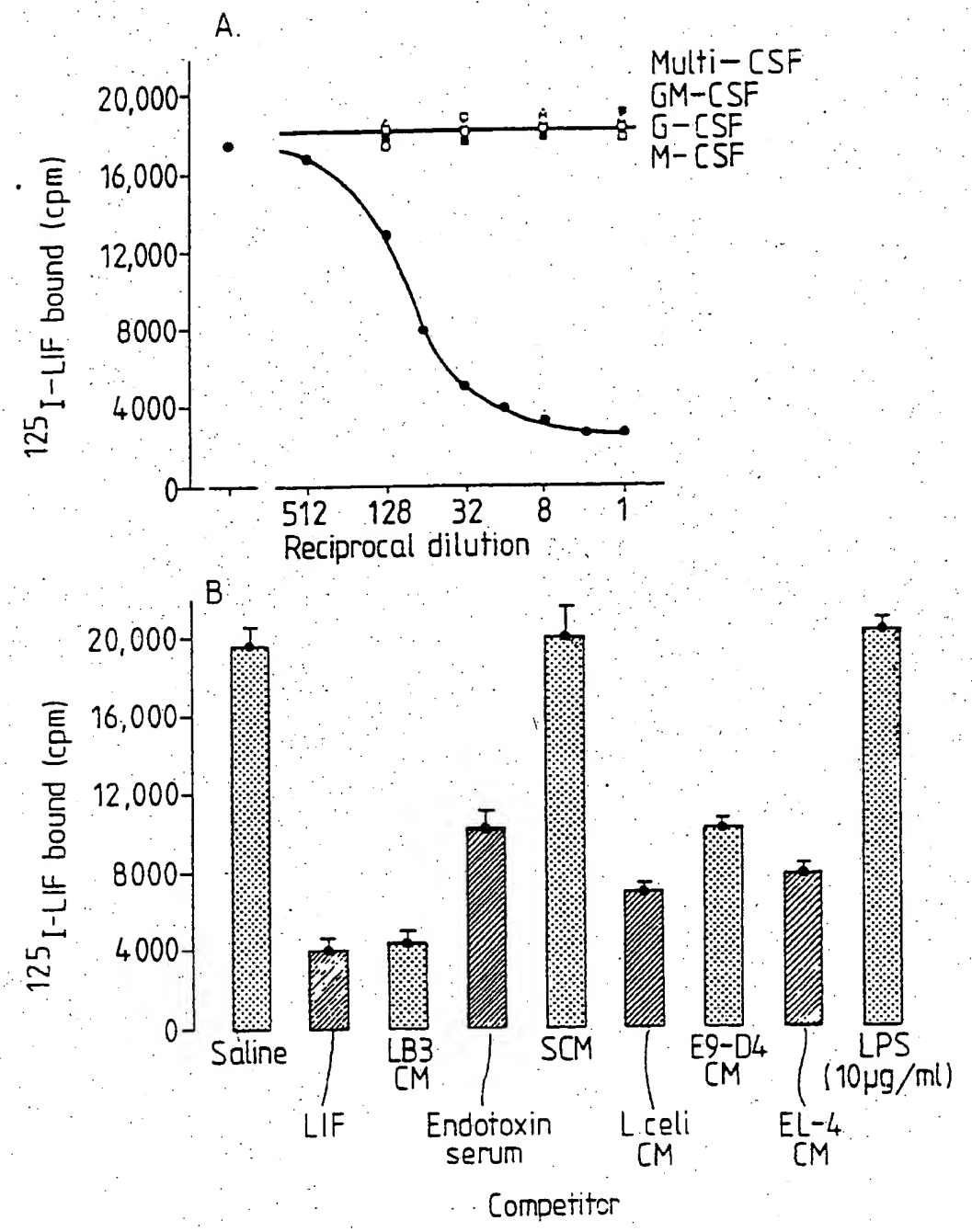

A.
Multi-CSF
GM-CSF
G-CSF
M-CSF
20,000
16,000
12,000
8000
4000
0
125I-LIF bound (cpm)
512
128
32
8
1
Reciprocal dilution
B
20,000
16,000
12,000
8000
4000
0
125I-LIF bound (cpm)
Saline
LIF
LB3 CM
Endotoxin serum
SCM
L celi CM
E9-D4 CM
EL-4 CM
LPS (10µg/ml)
Competitor

FIG. 6.

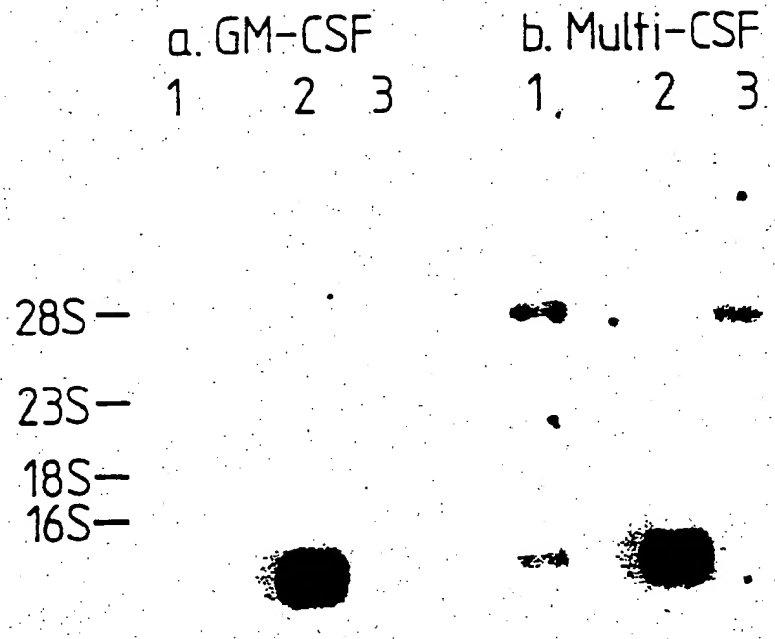


FIG. 7.

```
          15
          HisProCysHisGlyAsnLeuMetAsnGlnIleLys        LIF

                                  CUN           A
5'  CACCCNUGCCACGGNAACUUAAUGAACCAAAUCAAA  3'    LIF mRNA
      U     U U    U G     U G U G

3'  GTGGGGACGGTACCATTGGAGTACTTGGTCTAGTT  5'    LIF OLIGO
        A A   G    C          A
```

FIG. 8.

FIG. 9.

```
                                  ###                  ###          ***
HisTrpLysHisGlyAlaGlySerProLeuProIleThrProValAsnAl                              17
CACTGGAAACACGGGGCAGGGAGCCCTCTTCCCATCACCCCTGTAAATGC                              50

aThrCysAlaIleArgHisProCysHisGlyAsnLeuMetAsnGlnIleL                              34
CACCTGTGCCATACGCCACCCATGCCACGGCAACCTCATGAACCAGATCA                              100

                              ***
ysAsnGlnLeuAlaGlnLeuAsnGlySerAlaAsnAlaLeuPheIleSer                              50
AGAATCAACTGGCACAGCTCAATGGCAGCGCCAATGCTCTCTTCATTTCC                              150

TyrTyrThrAlaGlnGlyGluProPheProAsnAsnValGluLysLeuCy                              67
TATTACACAGCTCAAGGAGAGCCGTTTCCCAACAACGTGGAAAAGCTATG                              200

          ***   ###                                 ***
sAlaProAsnMetThrAspPheProSerPheHisGlyAsnGlyThrGluL                              84
TGCGCCTAACATGACAGACTTCCCATCTTTCCATGGCAACGGGACAGAGA                              250

ysThrLysLeuValGluLeuTyrArgMetValAlaTyrLeuSerAlaSer                              100
AGACCAAGTTGGTGGAGCTGTATCGGATGGTCGCATACCTGAGCGCCTCC                              300

      ***                                   ***   ###
LeuThrAsnIleThrArgAspGlnLysValLeuAsnProThrAlaValSe                              117
CTGACCAATATCACCCGGGACCAGAAGGTCCTGAACCCCACTGCCGTGAG                              350

                          ***
rLeuGlnValLysLeuAsnAlaThrIleAspValMetArgGlyLeuLeuS                              134
CCTCCAGGTCAAGCTCAATGCTACTATAGACGTCATGAGGGGCCTCCTCA                              400

erAsnValLeuCysArgLeuCysAsnLysTyrArgValGlyHisValAsp                              150
GCAATGTGCTTTGCCGTCTGTGCAACAAGTACCGTGTGGGCCACGTGGAT                              450

ValProProValProAspHisSerAspLysGluAlaPheGlnArgLysLy                              167
GTGCCACCTGTCCCCGACCACTCTGACAAAGAAGCCTTCCAAAGGAAAAA                              500

sLeuGlyCysGlnLeuLeuGlyThrTyrLysGln                                              178
GTTGGGTTGCCAGCTTCTGGGGACATACAAGCAAG                                             535
```

FIG. 10.

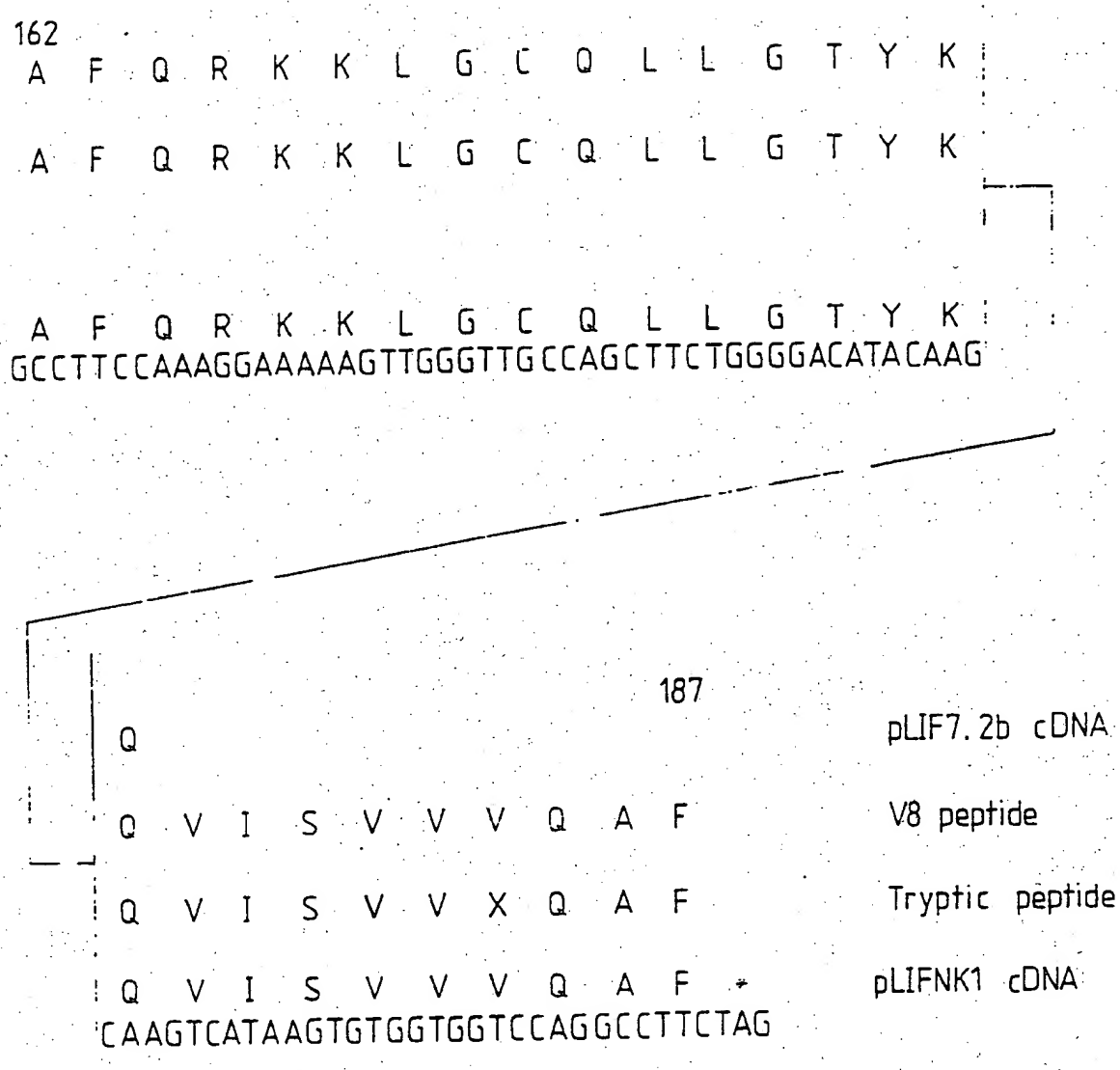

162
A F Q R K K L G C Q L L G T Y K
A F Q R K K L G C Q L L G T Y K
A F Q R K K L G C Q L L G T Y K
GCCTTCCAAAGGAAAAAGTTGGGTTGCCAGCTTCTGGGGACATACAAG
187
Q
pLIF7.2b cDNA
Q V I S V V V Q A F
V8 peptide
Q V I S V V X Q A F
Tryptic peptide
Q V I S V V V Q A F *
pLIFNK1 cDNA
CAAGTCATAAGTGTGGTGGTCCAGGCCTTCTAG

FIG. 11.

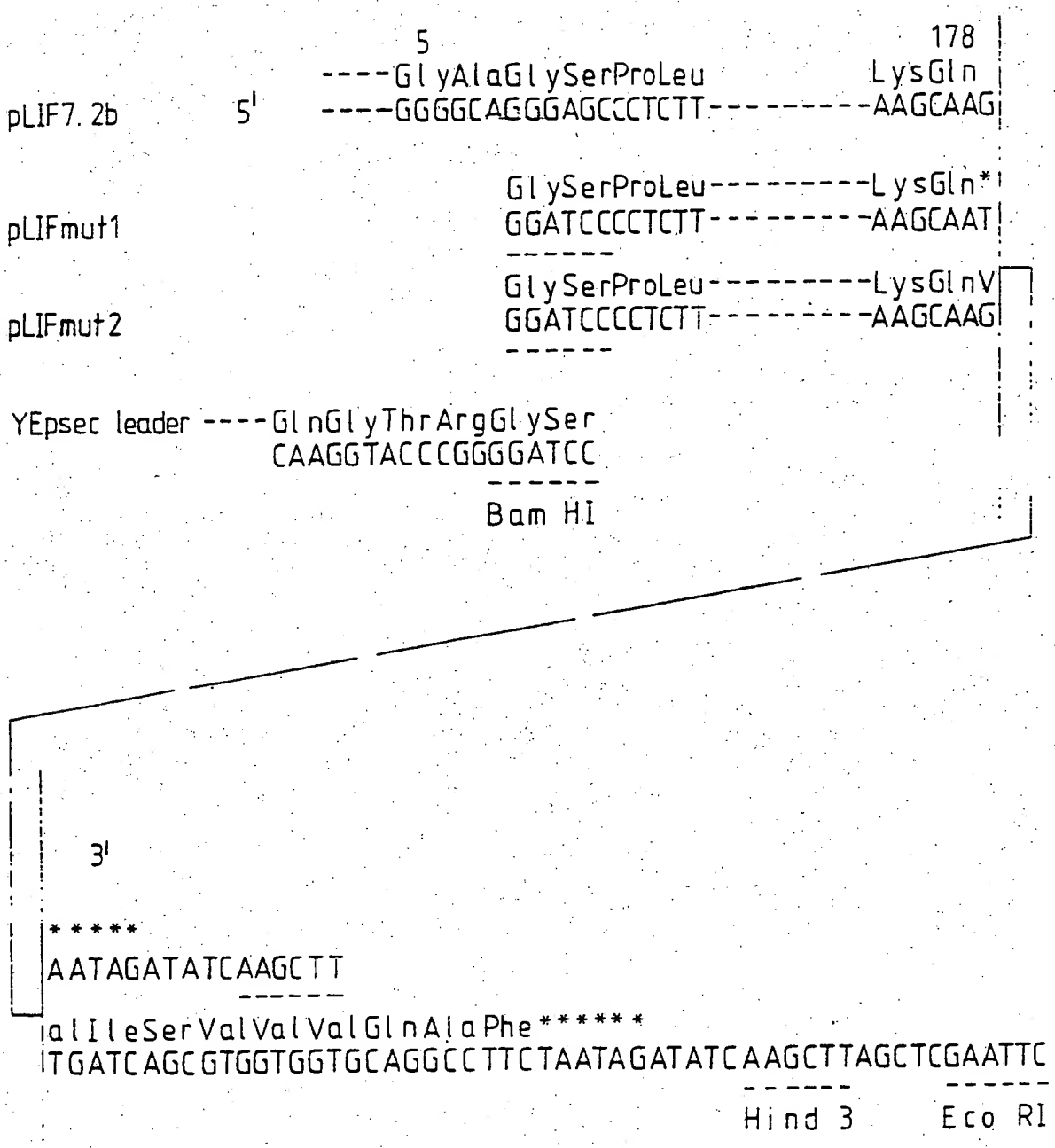

5
178
GlyAlaGlySerProLeu
LysGln
pLIF7.2b
5'
GGGGCAGGGAGCCCTCTT
AAGCAAG
GlySerProLeu
LysGln*
pLIFmut1
GGATCCCCTCTT
AAGCAAT
GlySerProLeu
LysGlnV
pLIFmut2
GGATCCCCTCTT
AAGCAAG
YEpsec leader
GlnGlyThrArgGlySer
CAAGGTACCCGGGGATCC
Bam HI
3'
*****
AATAGATATCAAGCTT
alIleSerValValValGlnAlaPhe******
TGATCAGCGTGGTGGTGCAGGCCTTCTAATAGATATCAAGCTTAGCTCGAATTC
Hind 3
Eco RI

FIG.12.

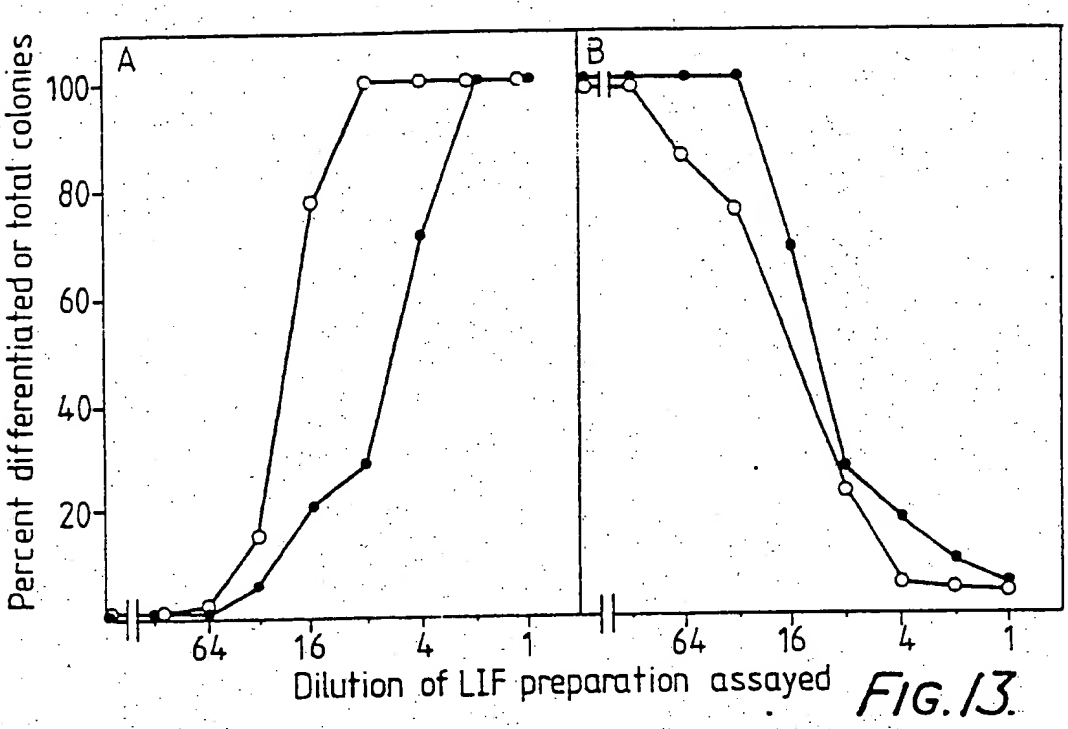

A
B
Percent differentiated or total colonies
100
80
60
40
20
64
16
4
1
Dilution of LIF preparation assayed

FIG. 13.

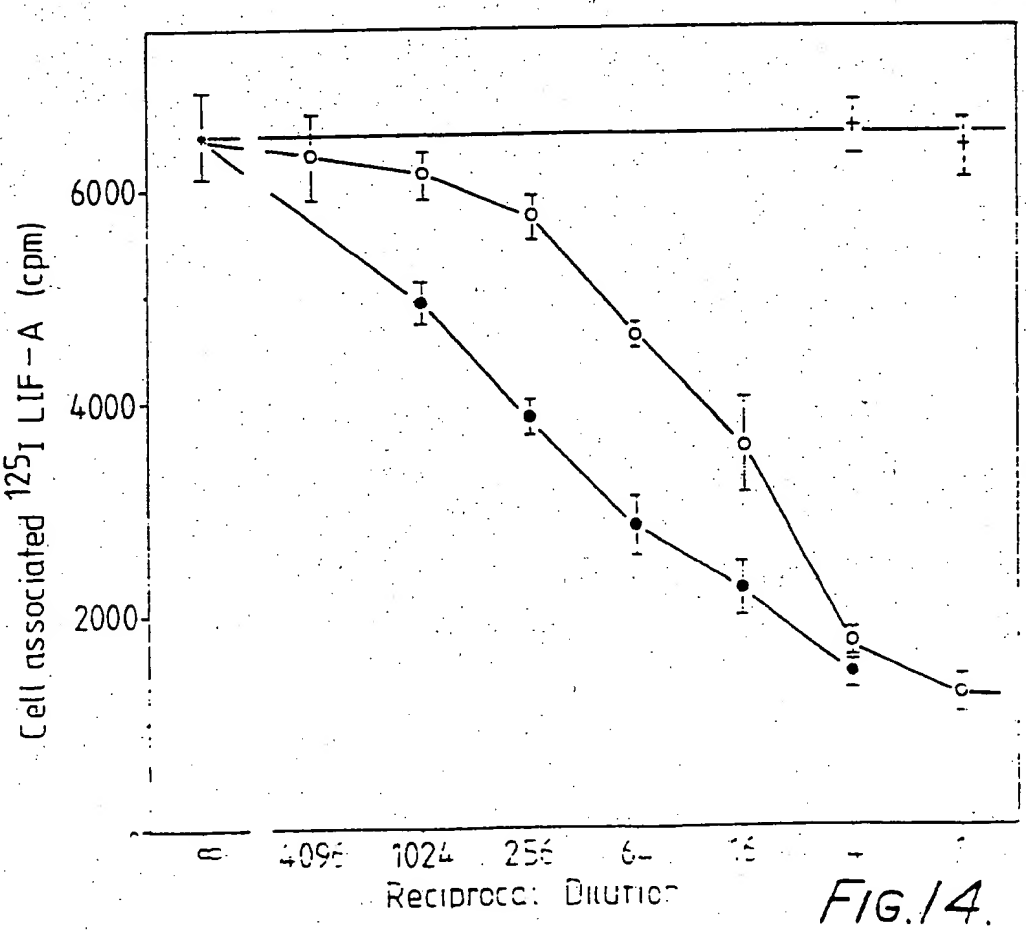

Cell associated ^{125}I LIF-A (cpm)
6000
4000
2000
∞
4096
1024
256
64
16
4
1
Reciprocal Dilution

FIG. 14.

13/35

```
EcoRI                                   MetLysValLeuAlaAlaGlyIleValP
GAATTCCGGAGTCCAGCCCATAATGAAGGTCTTGGCCGCAGGGATTGTGC                    50
------                                                    +1

roLeuLeuLeuLeuValLeuHisTrpLysHisGlyAlaGlySerProLeu
CCTTACTGCTGCTGGTTCTGCACTGGAAACACGGGGCAGGGAGCCCTCTT                   100

ProIleThrProValAsnAlaThrCysAlaIleArgHisProCysHisGl
CCCATCACCCCTGTAAATGCCACCTGTGCCATACGCCACCCATGCCACGG                   150

yAsnLeuMetAsnGlnIleLysAsnGlnLeuAlaGlnLeuAsnGlySerA
CAACCTCATGAACCAGATCAAGAATCAACTGGCACAGCTCAATGGCAGCG                   200

laAsnAlaLeuPheIleSerTyrTyrThrAlaGlnGlyGluProPhePro
CCAATGCTCTCTTCATTTCCTATTACACAGCTCAAGGAGAGCCGTTTCCC                   250

AsnAsnValGluLysLeuCysAlaProAsnMetThrAspPheProSerPh
AACAACGTGGAAAAGCTATGTGCGCCTAACATGACAGACTTCCCATCTTT                   300

eHisGlyAsnGlyThrGluLysThrLysLeuValGluLeuTyrArgMetV
CCATGGCAACGGGACAGAGAAGACCAAGTTGGTGGAGCTGTATCGGATGG                   350

alAlaTyrLeuSerAlaSerLeuThrAsnIleThrArgAspGlnLysVal
TCGCATACCTGAGCGCCTCCCTGACCAATATCACCCGGGACCAGAAGGTC                   400

LeuAsnProThrAlaValSerLeuGlnValLysLeuAsnAlaThrIleAs
CTGAACCCCACTGCCGTGAGCCTCCAGGTCAAGCTCAATGCTACTATAGA                   450

pValMetArgGlyLeuLeuSerAsnValLeuCysArgLeuCysAsnLysT
CGTCATGAGGGGCCTCCTCAGCAATGT-CTTTGCCGTCTGTGCAACAAGT                   500

yrArgValGlyHisValAspValProProValProAspHisSerAspLys
ACCGTGTGGGCCACGTGGATGTGCCACCTGTCCCCGACCACTCTGACAAA                    550
```

FIG. 15A.

```
GluAlaPheGlnArgLysLysLeuGlyCysGlnLeuLeuGlyThrTyrLy
GAAGCCTTCCAAAGGAAAAAGTTGGGTTGCCAGCTTCTGGGGACATACAA   600

sGlnValIleSerValValValGlnAlaPhe***
GCAAGTCATAAGTGTGGTGGTCCAGGCCTTCTAGAGAGGAGGTCTTGAAT   650
                                  ------
                                  xba I
GTACCATGGACTGAGGGACCTCAGGAGCAGGATCCGGAGGTGGGGAGGGG   700
GCTCAAAATGTGCTGGGGTTTGGGACATTGTTAAATGCAAAACGGGGCTG   750
CTGGCAGACCCCAGGGATTTCCAGGTACTCACTGCACTCTGGGCTGGGCC   800
ATGATGGAATCTGGCAAAGTTGAAACTTCCATAGGCAGAGCTTCTATACA   850
GCCCAGCACCAGCTAGAAATGGCAATGAGGGTGTTGGTCTGAGAGATTTC   900
TGTCTCACTCACTCACTCACTCACTCTCACTCACTCACTCACTCACTCAC   950
TCAGCCCCTTGCTTGCTGGGTGTAGAACAAGCTGCCACAAGTTGTCTACA  1000
GCAGACAGCAAAGGGCTGGGAAGTGTCCTAGACCCCTACAGAGTCACCAT  1050
CATCTGGTCCTTTGCTGTCTCTCAGAGAAACTTTGGAAGGCTTGGTTGGG  1100
ATGTGAGAGAGCTAAGGGGACTGGGATCCAGAAGGAATCCTTTTATTTTA  1150
TTTTATTTTATTTTATTTTATTTTATTTTATAAGTTTTGTGGGTGGAAGG  1200
GTACCCTGGGGTGGAATGATGGAATGTGTCTTCTCTTGAGTTGGATGAGA  1250
GAGTTCAGGCTTAGAGACTGTCAGATGGAAGAGTCTACCTCACCAGTGTT  1300
CAGCTCCCACAGAAGCACAGCGGCCAGCTTCCAGTTGTCAAAGCCT-ACG  1350
AACTCGGTTAGCTTCTATGCAGTTCCCCCCACAGCCTGGCGTGGTTGGGG  1400
TCTGCCAGCTGGACCTAGAGGTGAGGTGTGTGGAATTC              1438
```

FIG. 15B.

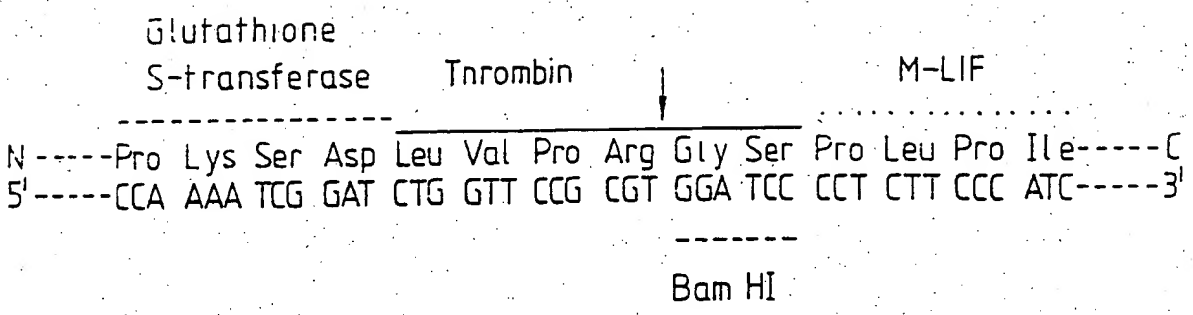


FIG.16.

(a) 5'-CTGCACTGGACCTTAGGGGCGGGATCCCCCCTCCCCATC-3'
Bam HI

(b) 5'-GCCAATGCCCTCTTTATTCTCTATTACACAGCCCAGGGGGAGCCGTTC-3'

(c) 5'-CAGGCCTTCTAGTAAGATATCAAGCTTTGTGCTGTGAAC-3'
Hind 3

FIG.28.

16/35

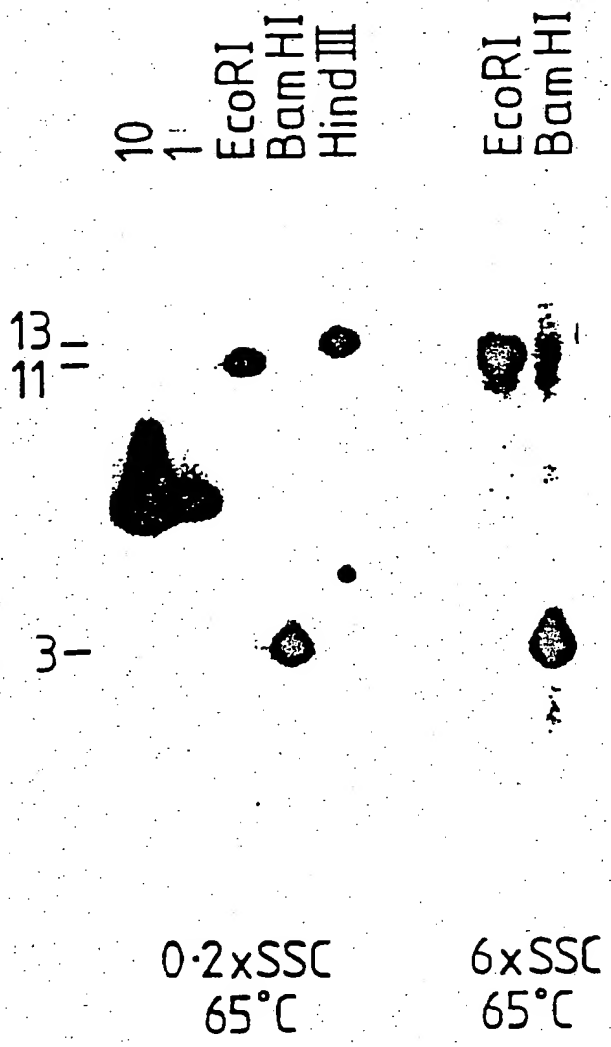

10
1
EcoRI
BamHI
HindIII
EcoRI
BamHI
13
11
3
0·2xSSC
65°C
6xSSC
65°C

FIG. 17.

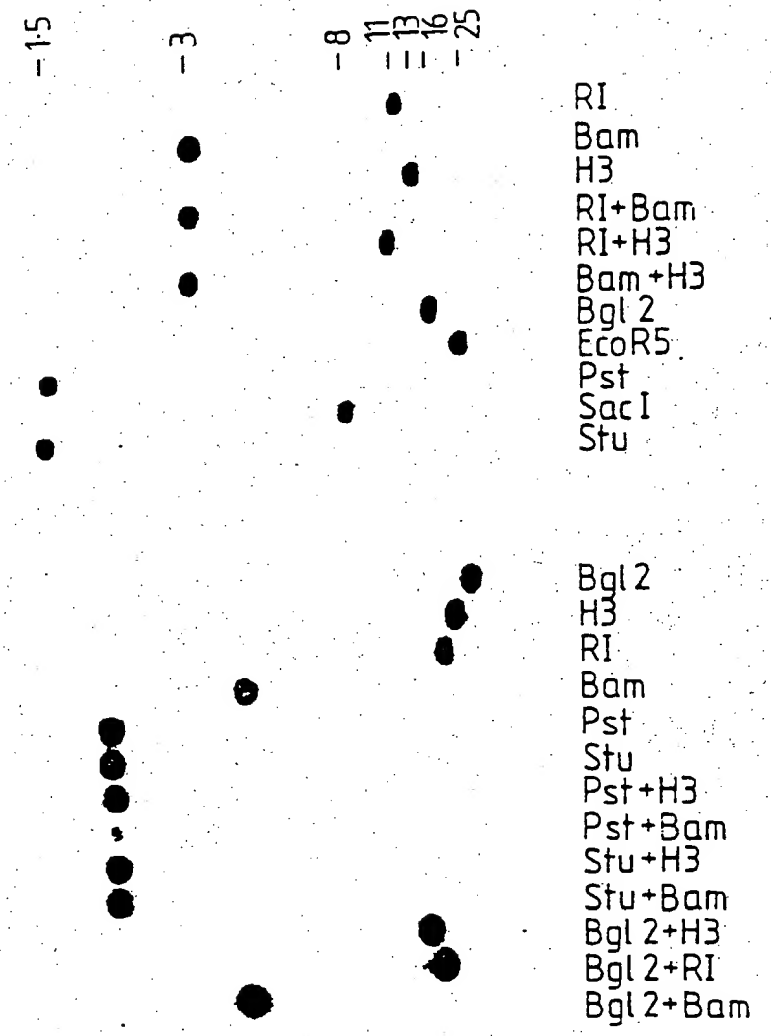

-15
-3
-8
-11
-13
-16
-25
RI
Bam
H3
RI+Bam
RI+H3
Bam+H3
Bgl 2
EcoR5
Pst
Sac I
Stu
Bgl 2
H3
RI
Bam
Pst
Stu
Pst+H3
Pst+Bam
Stu+H3
Stu+Bam
Bgl 2+H3
Bgl 2+RI
Bgl 2+Bam

FIG. 18.

18/35

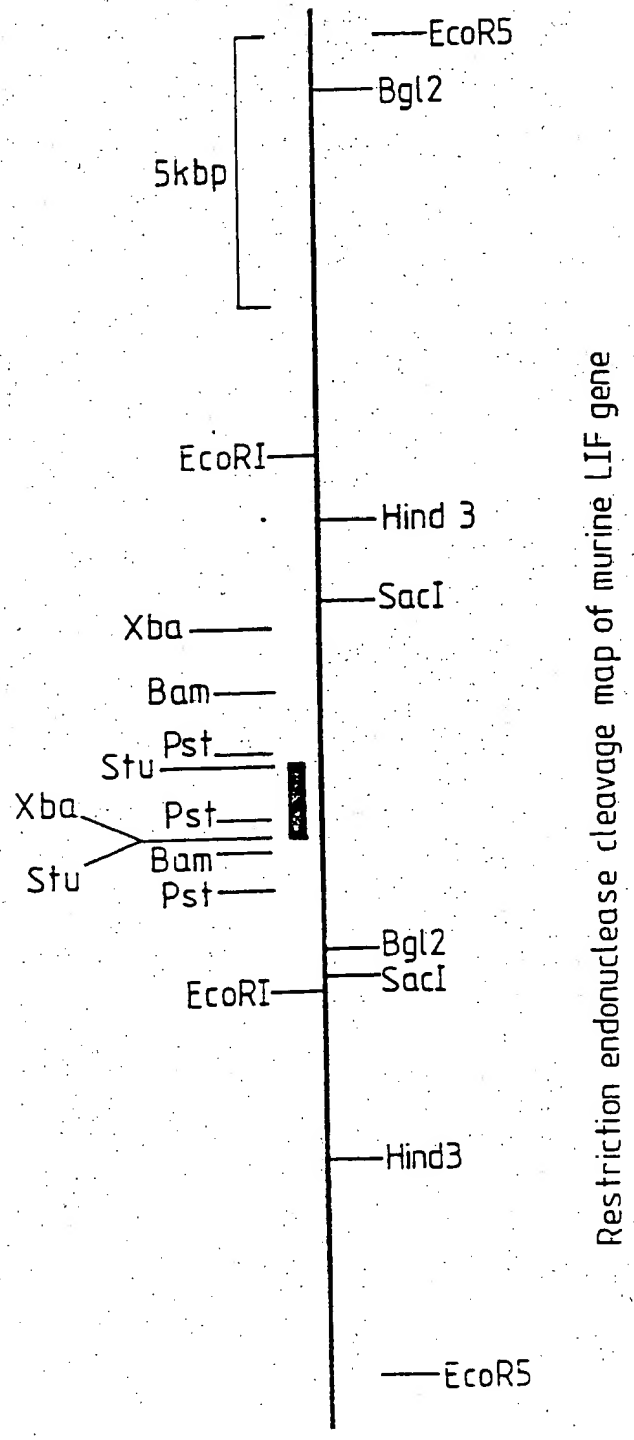

EcoR5
Bgl2
5kbp
EcoRI
Hind 3
SacI
Xba
Bam
Pst
Stu
Xba
Pst
Bam
Stu
Pst
Bgl2
SacI
EcoRI
Hind3
EcoR5
Restriction endonuclease cleavage map of murine LIF gene

FIG. 19.

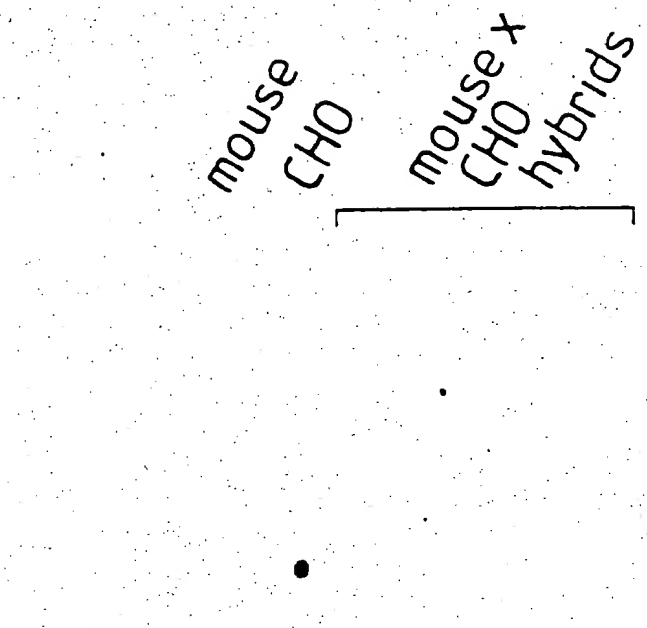


FIG. 20.

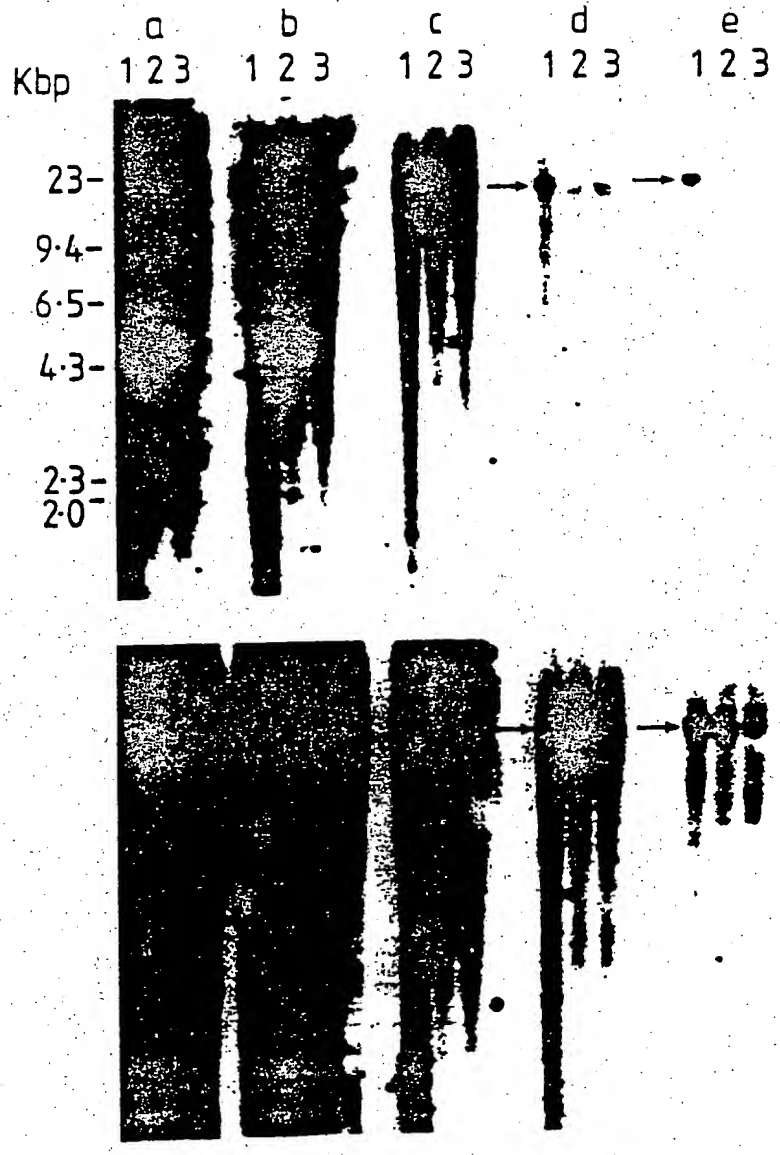
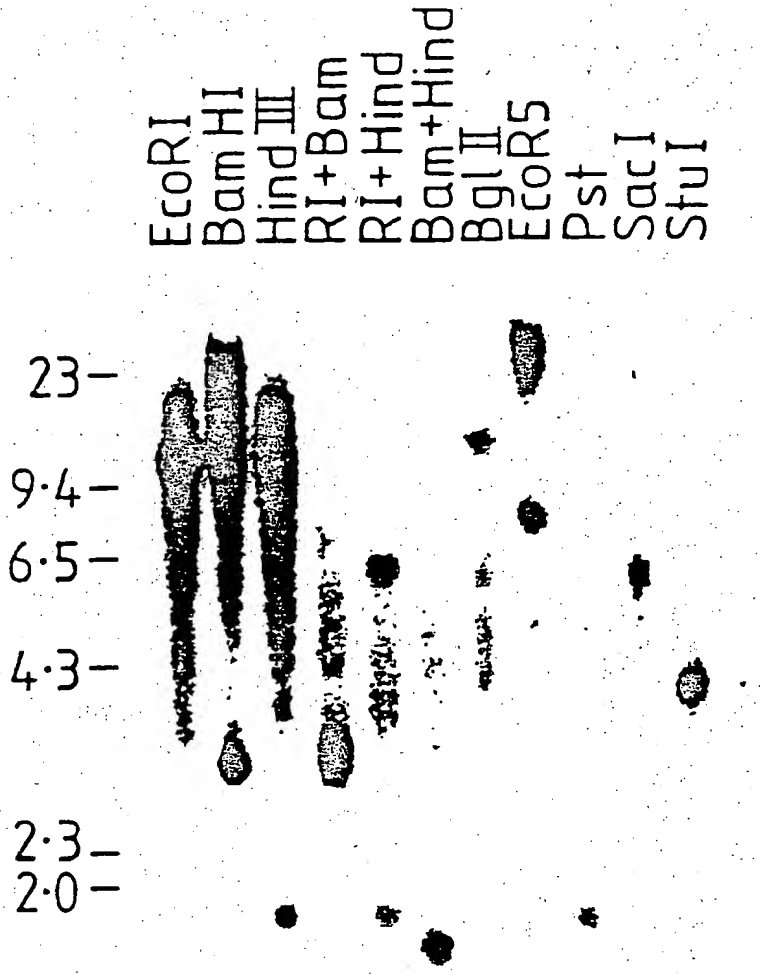


FIG. 21.



6xSSC 65°C

FIG. 22.

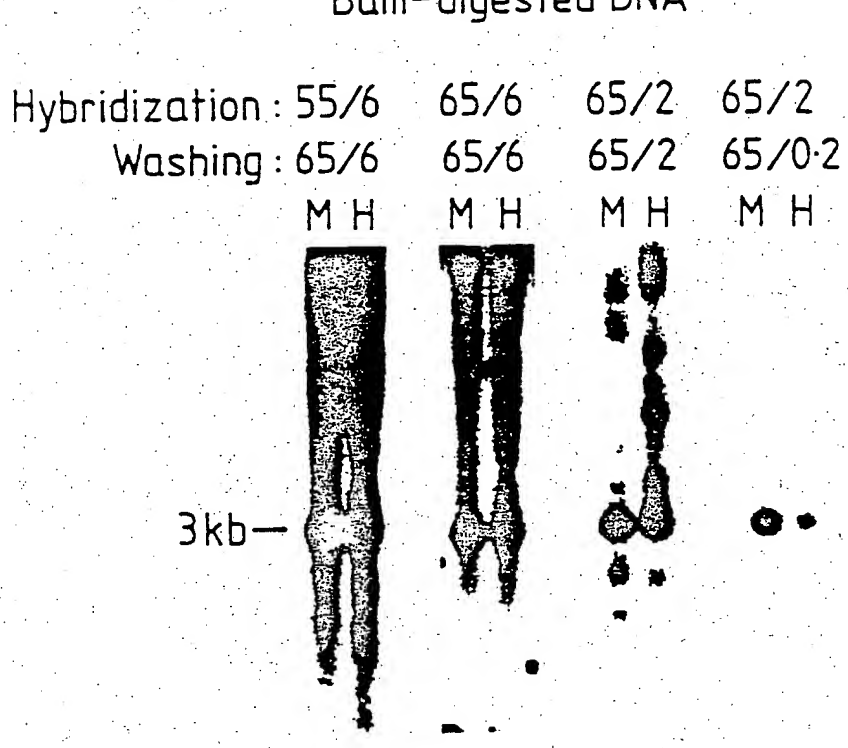

Bam-digested DNA
Hybridization : 55/6 65/6 65/2 65/2
Washing : 65/6 65/6 65/2 65/0·2
M H M H M H M H
3kb—

FIG. 23.

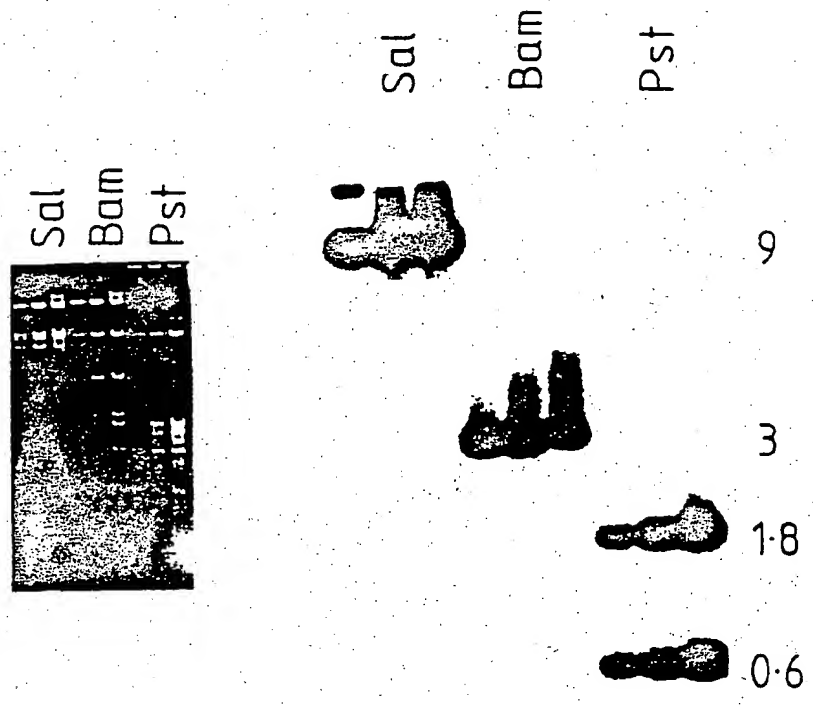


FIG. 24.

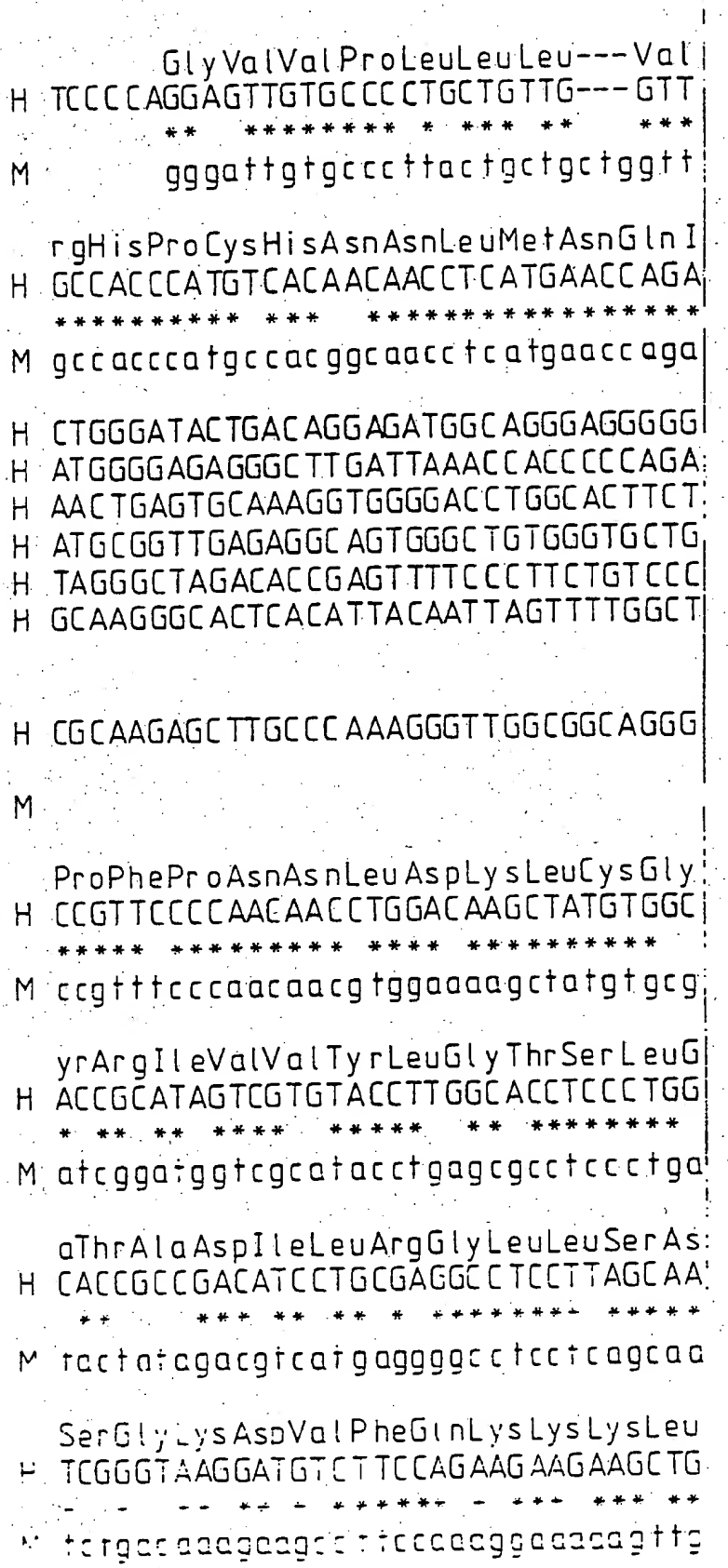

GlyValValProLeuLeuLeu---Val
H TCCCAGGAGTTGTGCCCCTGCTGTTG---GTT
M gggattgtgcccttactgctgctggtt
rgHisProCysHisAsnAsnLeuMetAsnGlnI
H GCCACCCATGTCACAACAACCTCATGAACCAGA
M gccacccatgccacggcaacctcatgaaccaga
H CTGGGATACTGACAGGAGATGGCAGGGAGGGGG
H ATGGGGAGAGGGCTTGATTAAACCACCCCAGA
H AACTGAGTGCAAAGGTGGGGACCTGGCACTTCT
H ATGCGGTTGAGAGGCAGTGGGCTGTGGGTGCTG
H TAGGGCTAGACACCGAGTTTTCCCTTCTGTCCC
H GCAAGGGCACTCACATTACAATTAGTTTTGGCT
H CGCAAGAGCTTGCCCAAAGGGTTGGCGGCAGGG
M
ProPheProAsnAsnLeuAspLysLeuCysGly
H CCGTTCCCCAACAACCTGGACAAGCTATGTGGC
M ccgtttcccaacaacgtggaaaagctatgtgcg
yrArgIleValValTyrLeuGlyThrSerLeuG
H ACCGCATAGTCGTGTACCTTGGCACCTCCCTGG
M atcggatggtcgcatacctgagcgcctccctga
aThrAlaAspIleLeuArgGlyLeuLeuSerAs
H CACCGCCGACATCCTGCGAGGCCTCCTTAGCAA
M tactatcgacgtcatgaggggcctcctcagcaa
SerGlyLysAspValPheGlnLysLysLysLeu
H TCGGGTAAGGATGTCTTCCAGAAGAAGAAGCTG
M tcrgccaaagcagcctttcccacggaaaaagttg

FIG. 25A.

25/35

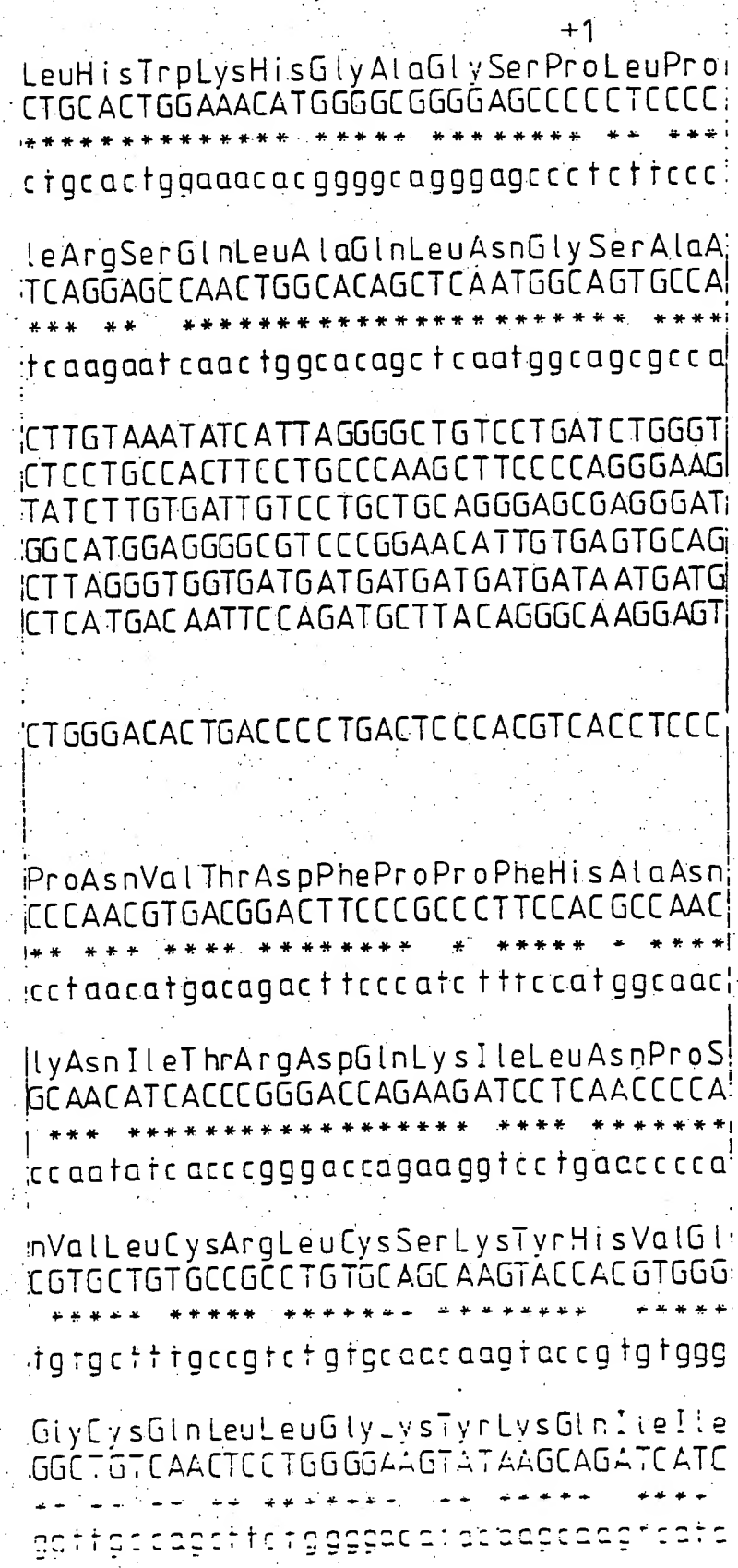

+1
LeuHisTrpLysHisGlyAlaGlySerProLeuPro
CTGCACTGGAAACATGGGGCGGGGAGCCCCTCCCC
ctgcactggaaacacggggcagggagccctcttccc
leArgSerGlnLeuAlaGlnLeuAsnGlySerAlaA
TCAGGAGCCAACTGGCACAGCTCAATGGCAGTGCCA
tcaagaatcaactggcacagctcaatggcagcgcca
CTTGTAAATATCATTAGGGGCTGTCCTGATCTGGGT
CTCCTGCCACTTCCTGCCCAAGCTTCCCCAGGGAAG
TATCTTGTGATTGTCCTGCTGCAGGGAGCGAGGGAT
GGCATGGAGGGGCGTCCCGGAACATTGTGAGTGCAG
CTTAGGGTGGTGATGATGATGATGATGATAATGATG
CTCATGACAATTCCAGATGCTTACAGGGCAAGGAGT
CTGGGACACTGACCCCTGACTCCCACGTCACCTCCC
ProAsnValThrAspPheProProPheHisAlaAsn
CCCAACGTGACGGACTTCCCGCCCTTCCACGCCAAC
cctaacatgacagacttcccatctttccatggcaac
lyAsnIleThrArgAspGlnLysIleLeuAsnProS
GCAACATCACCCGGGACCAGAAGATCCTCAACCCCA
ccaatatcacccgggaccagaaggtcctgaccccca
nValLeuCysArgLeuCysSerLysTyrHisValGl
CGTGCTGTGCCGCCTGTGCAGCAAGTACCACGTGGG
tgtgctttgccgtctgtgcaccaagtaccgtgtggg
GlyCysGlnLeuLeuGly_ysTyrLysGlnIleIle
GGCTGTCAACTCCTGGGGAAGTATAAGCAGATCATC
ggttgccagcttctgggggaccatcaaggcaagtcatc

FIG. 25B.

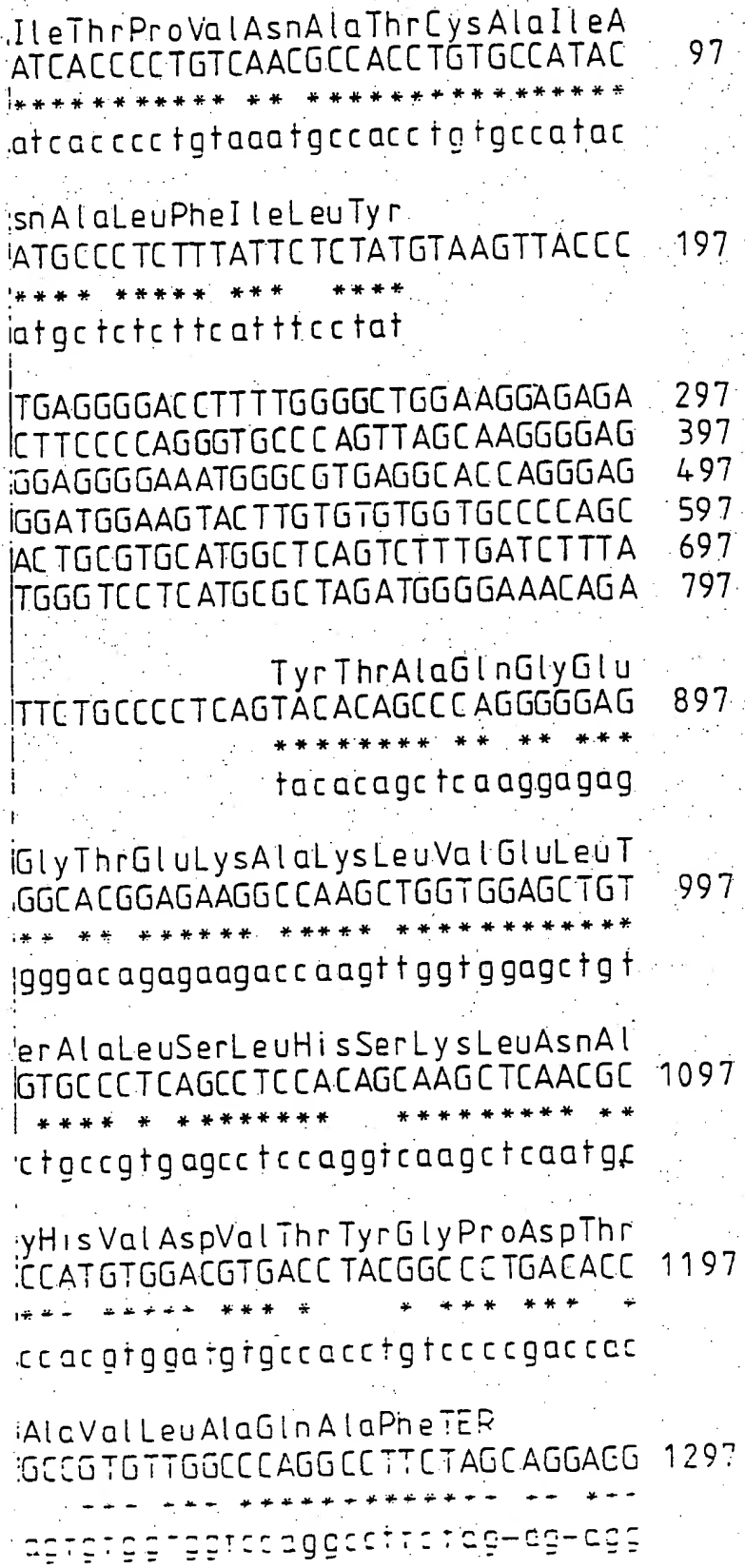

IleThrProValAsnAlaThrCysAlaIleA
ATCACCCCTGTCAACGCCACCTGTGCCATAC 97
atcaccctgtaaatgccacctgtgccatac
snAlaLeuPheIleLeuTyr
ATGCCCTCTTTATTCTCTATGTAAGTTACCC 197
atgctctcttcatttcctat
TGAGGGGACCTTTTGGGGCTGGAAGGAGAGA 297
CTTCCCCAGGGTGCCCAGTTAGCAAGGGGAG 397
GGAGGGGAAATGGGCGTGAGGCACCAGGGAG 497
GGATGGAAGTACTTGTGTGGTGCCCCAGC 597
ACTGCGTGCATGGCTCAGTCTTTGATCTTTA 697
TGGGTCCTCATGCGCTAGATGGGGAAACAGA 797
TyrThrAlaGlnGlyGlu
TTCTGCCCCTCAGTACACAGCCCAGGGGGAG 897
tacacagctcaaggagag
GlyThrGluLysAlaLysLeuValGluLeuT
GGCACGGAGAAGGCCAAGCTGGTGGAGCTGT 997
gggacagagaagaccaagttggtggagctgt
erAlaLeuSerLeuHisSerLysLeuAsnAl
GTGCCCTCAGCCTCCACAGCAAGCTCAACGC 1097
ctgccgtgagcctccaggtcaagctcaatgc
yHisValAspValThrTyrGlyProAspThr
CCATGTGGACGTGACCTACGGCCCTGACACC 1197
ccacgtggatgtgccacctgtccccgaccac
AlaValLeuAlaGlnAlaPheTER
GCCGTGTTGGCCCAGGCCTTCTAGCAGGACG 1297
agtgtggtggtccaggccttctag-gg-cgg

FIG. 25C.

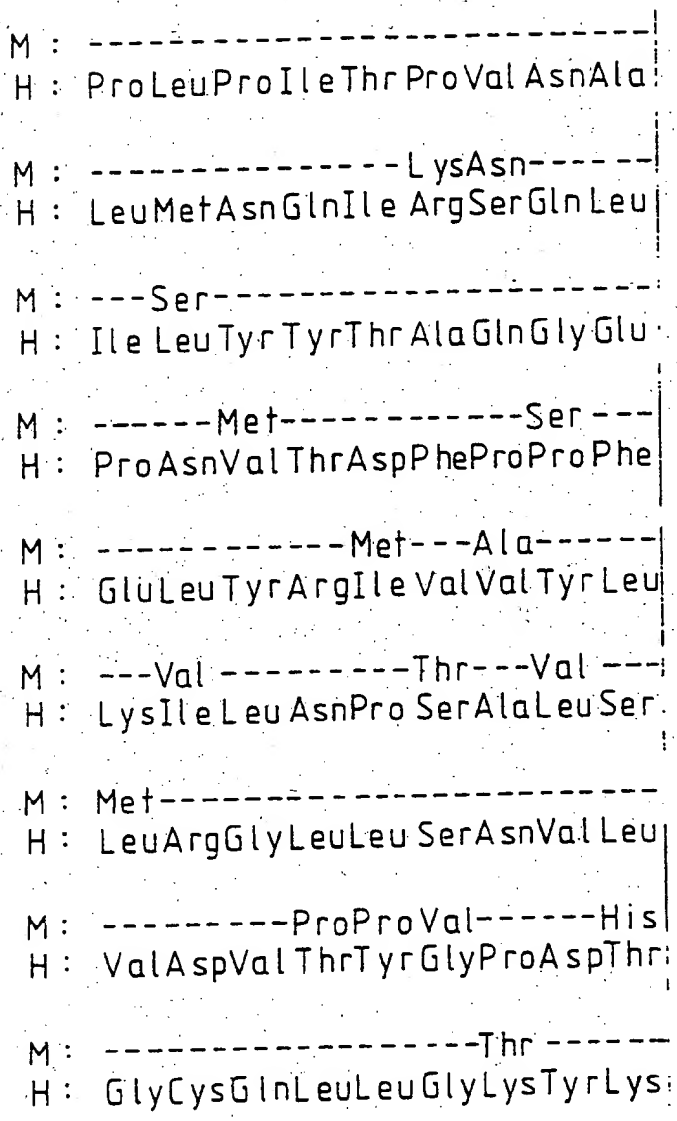

M : ----------
H : ProLeuProIleThrProValAsnAla
M : ----------LysAsn------
H : LeuMetAsnGlnIle ArgSerGlnLeu
M : ---Ser----------
H : Ile LeuTyr TyrThr AlaGlnGlyGlu
M : ------Met----------Ser---
H : ProAsnValThrAspPheProProPhe
M : ----------Met---Ala------
H : GluLeuTyrArgIle ValValTyrLeu
M : ---Val ---------Thr---Val ---
H : LysIle Leu AsnPro SerAlaLeuSer
M : Met----------
H : LeuArgGlyLeuLeu SerAsnVal Leu
M : ---------ProProVal------His
H : ValAspVal ThrTyrGlyProAspThr
M : ----------Thr ------
H : GlyCysGlnLeuLeuGlyLysTyrLys

FIG. 26A.

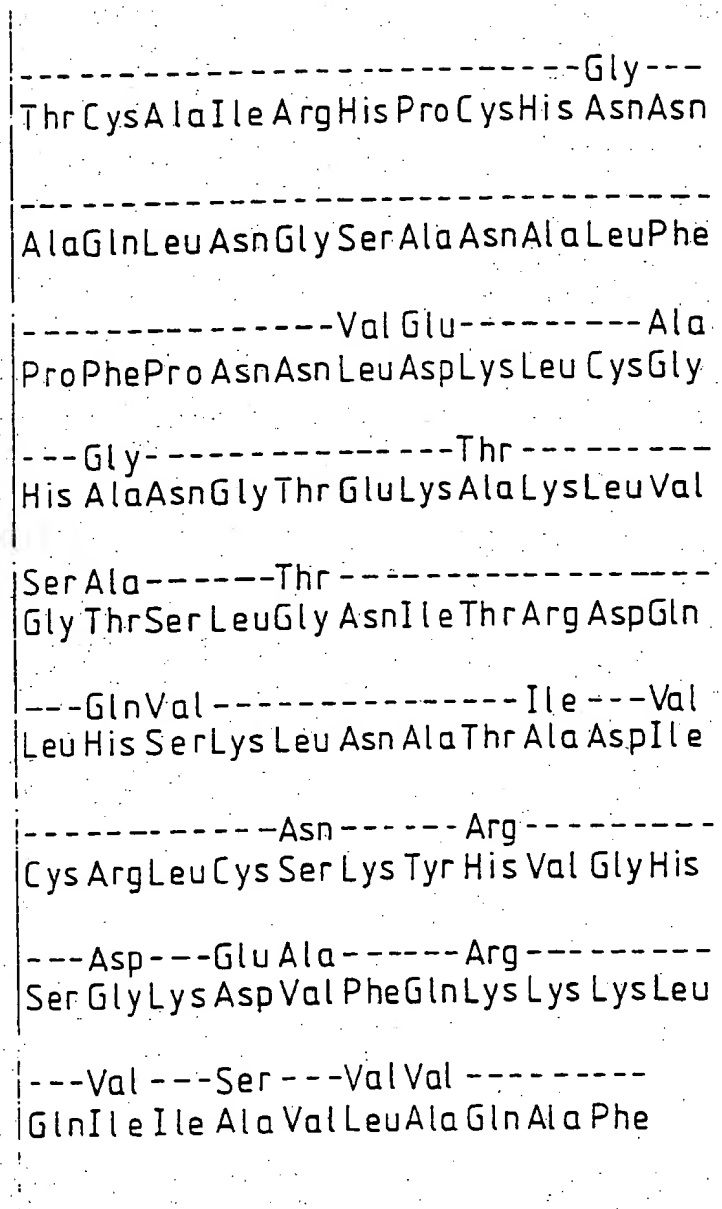

Gly
Thr Cys Ala Ile Arg His Pro Cys His Asn Asn
Ala Gln Leu Asn Gly Ser Ala Asn Ala Leu Phe
Val Glu Ala
Pro Phe Pro Asn Asn Leu Asp Lys Leu Cys Gly
Gly Thr
His Ala Asn Gly Thr Glu Lys Ala Lys Leu Val
Ser Ala Thr
Gly Thr Ser Leu Gly Asn Ile Thr Arg Asp Gln
Gln Val Ile Val
Leu His Ser Lys Leu Asn Ala Thr Ala Asp Ile
Asn Arg
Cys Arg Leu Cys Ser Lys Tyr His Val Gly His
Asp Glu Ala Arg
Ser Gly Lys Asp Val Phe Gln Lys Lys Lys Leu
Val Ser Val Val
Gln Ile Ile Ala Val Leu Ala Gln Ala Phe

FIG. 26B.

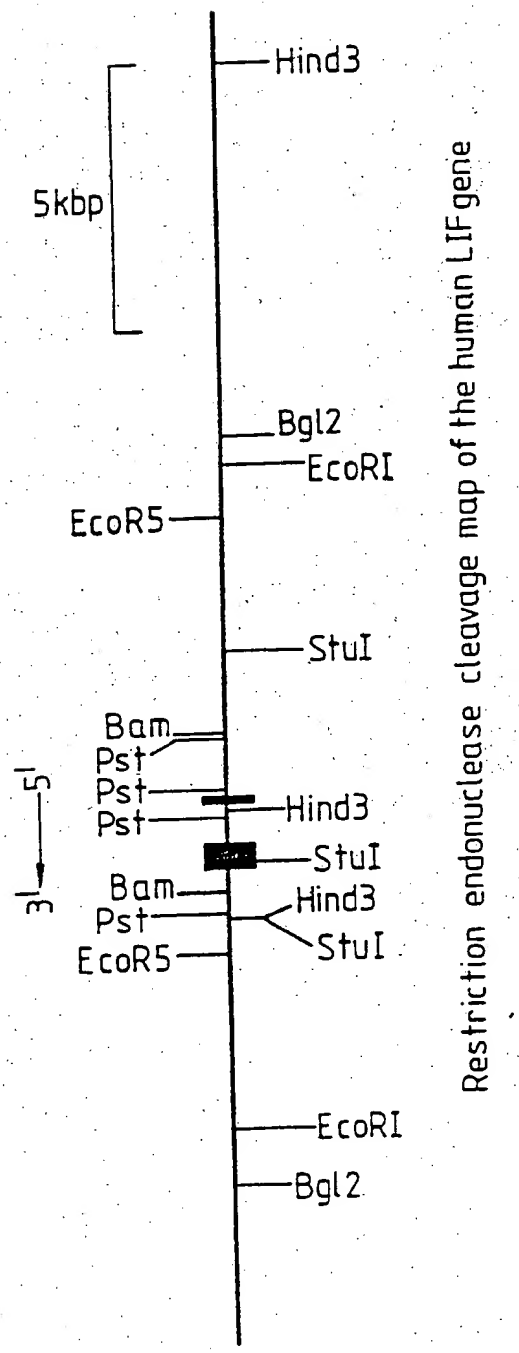

Hind3
5kbp
Bgl2
EcoRI
EcoR5
StuI
Bam
Pst
Pst
Hind3
Pst
StuI
5'
3'
Bam
Hind3
Pst
StuI
EcoR5
EcoRI
Bgl2
Restriction endonuclease cleavage map of the human LIF gene

FIG. 27.

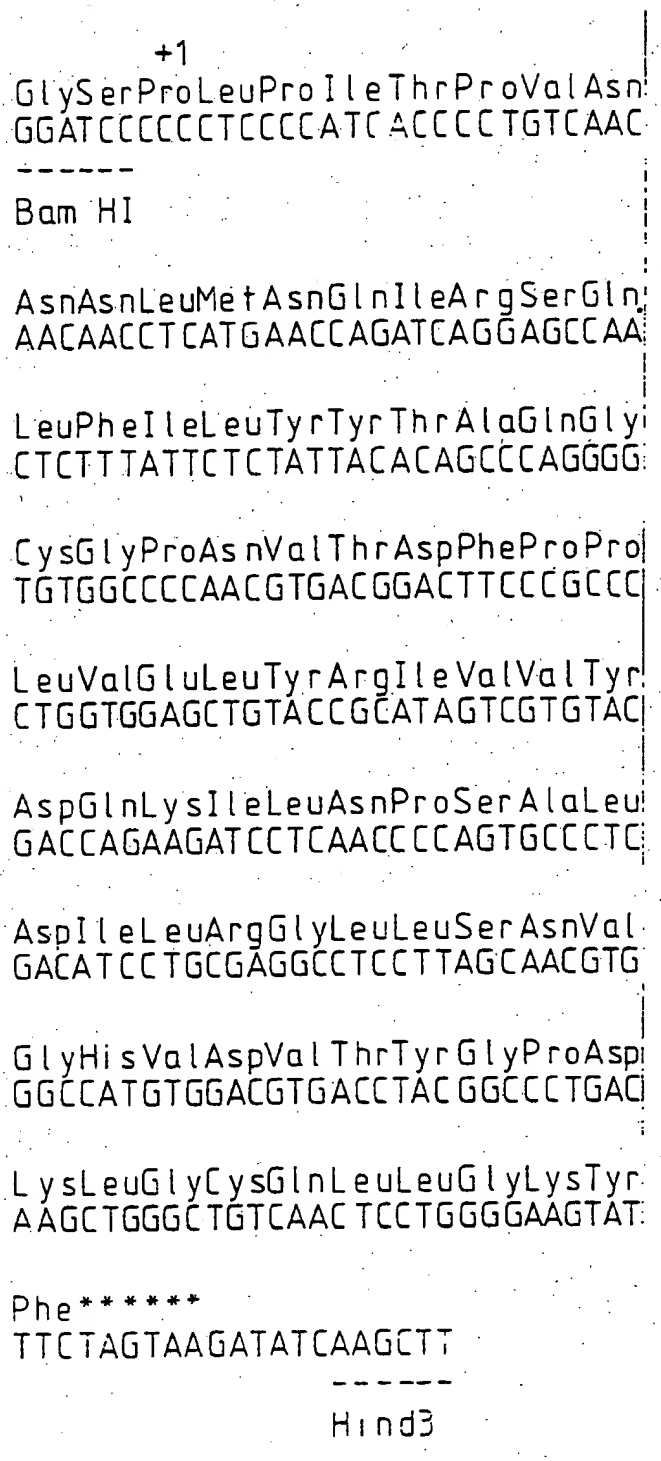

+1
GlySerProLeuProIleThrProValAsn
GGATCCCCCCTCCCCATCACCCCTGTCAAC
Bam HI
AsnAsnLeuMetAsnGlnIleArgSerGln
AACAACCTCATGAACCAGATCAGGAGCCAA
LeuPheIleLeuTyrTyrThrAlaGlnGly
CTCTTTATTCTCTATTACACAGCCCAGGGG
CysGlyProAsnValThrAspPheProPro
TGTGGCCCCAACGTGACGGACTTCCCGCCC
LeuValGluLeuTyrArgIleValValTyr
CTGGTGGAGCTGTACCGCATAGTCGTGTAC
AspGlnLysIleLeuAsnProSerAlaLeu
GACCAGAAGATCCTCAACCCCAGTGCCCTC
AspIleLeuArgGlyLeuLeuSerAsnVal
GACATCCTGCGAGGCCTCCTTAGCAACGTG
GlyHisValAspValThrTyrGlyProAsp
GGCCATGTGGACGTGACCTACGGCCCTGAC
LysLeuGlyCysGlnLeuLeuGlyLysTyr
AAGCTGGGCTGTCAACTCCTGGGGAAGTAT
Phe******
TTCTAGTAAGATATCAAGCTT
Hind3

FIG. 29A.

```
AlaThrCysAlaIleArgHisProCysHis
GCCACCTGTGCCATACGCCACCCATGTCAC   60

LeuAlaGlnLeuAsnGlySerAlaAsnAla
CTGGCACAGCTCAATGGCAGTGCCAATGCC  120

GluProPheProAsnAsnLeuAspLysLeu
GAGCCGTTCCCCAACAACCTGGACAAGCTA  180

PheHisAlaAsnGlyThrGluLysAlaLys
TTCCACGCCAACGGCACGGAGAAGGCCAAG  240

LeuGlyThrSerLeuGlyAsnIleThrArg
CTTGGCACCTCCCTGGGCAACATCACCCGG  300

SerLeuHisSerLysLeuAsnAlaThrAla
AGCCTCCACAGCAAGCTCAACGCCACCGCC  360

LeuCysArgLeuCysSerLysTyrHisVal
CTGTGCCGCCTGTGCAGCAAGTACCACGTG  420

ThrSerGlyLysAspValPheGlnLysLys
ACCTCGGGTAAGGATGTCTTCCAGAAGAAG  480

LysGlnIleIleAlaValLeuAlaGlnAla
AAGCAGATCATCGCCGTGTTGGCCCAGGCC  540

                                561
```

FIG. 29B.

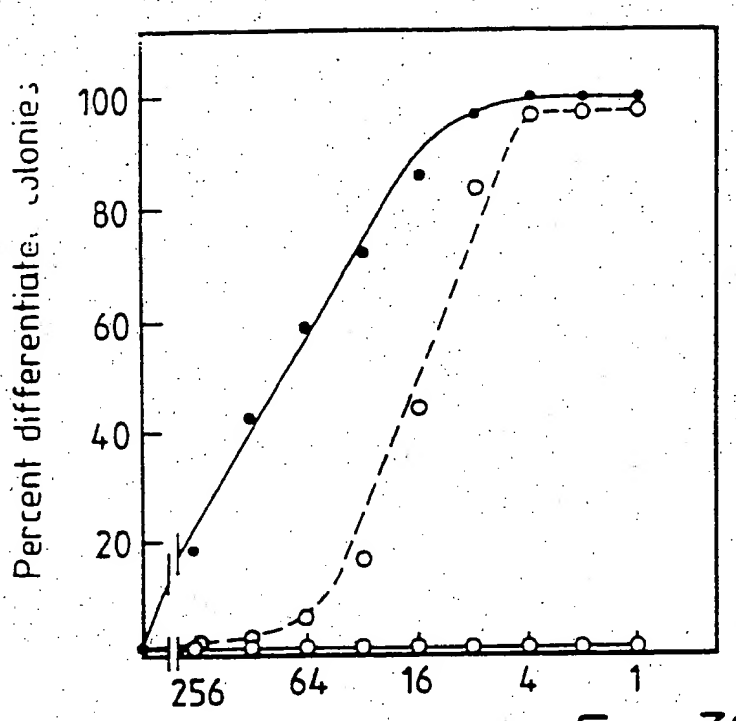

Percent differentiated colonies
100
80
60
40
20
256
64
16
4
1
Dilution added

FIG. 30.

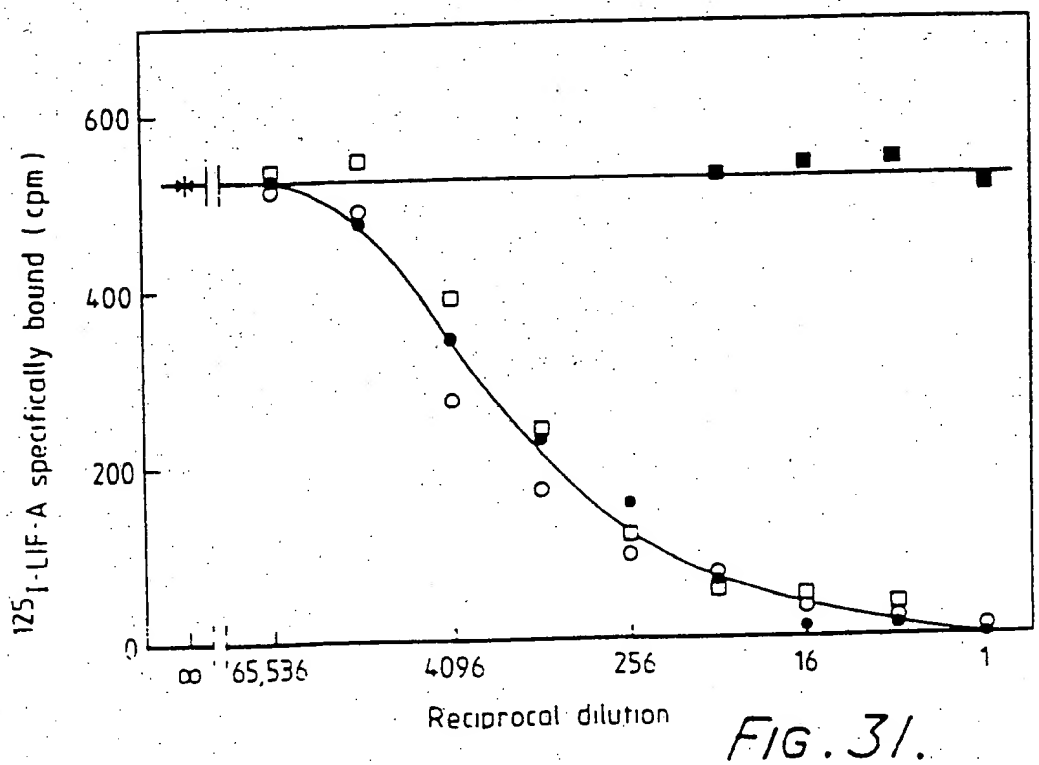

^{125}I-LIF-A specifically bound (cpm)
600
400
200
0
∞
65,536
4096
256
16
1
Reciprocal dilution

FIG. 31.

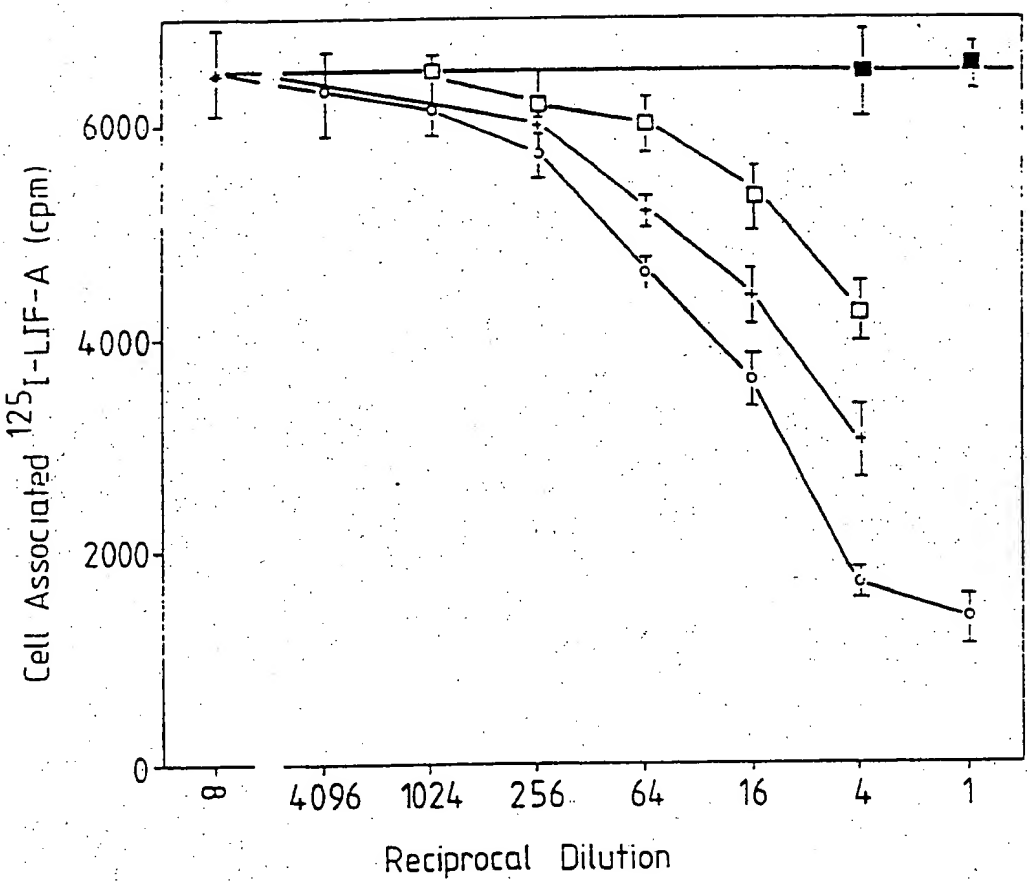

Cell Associated ^{125}I-LIF-A (cpm)
6000
4000
2000
0
∞
4096
1024
256
64
16
4
1
Reciprocal Dilution

FIG. 32.

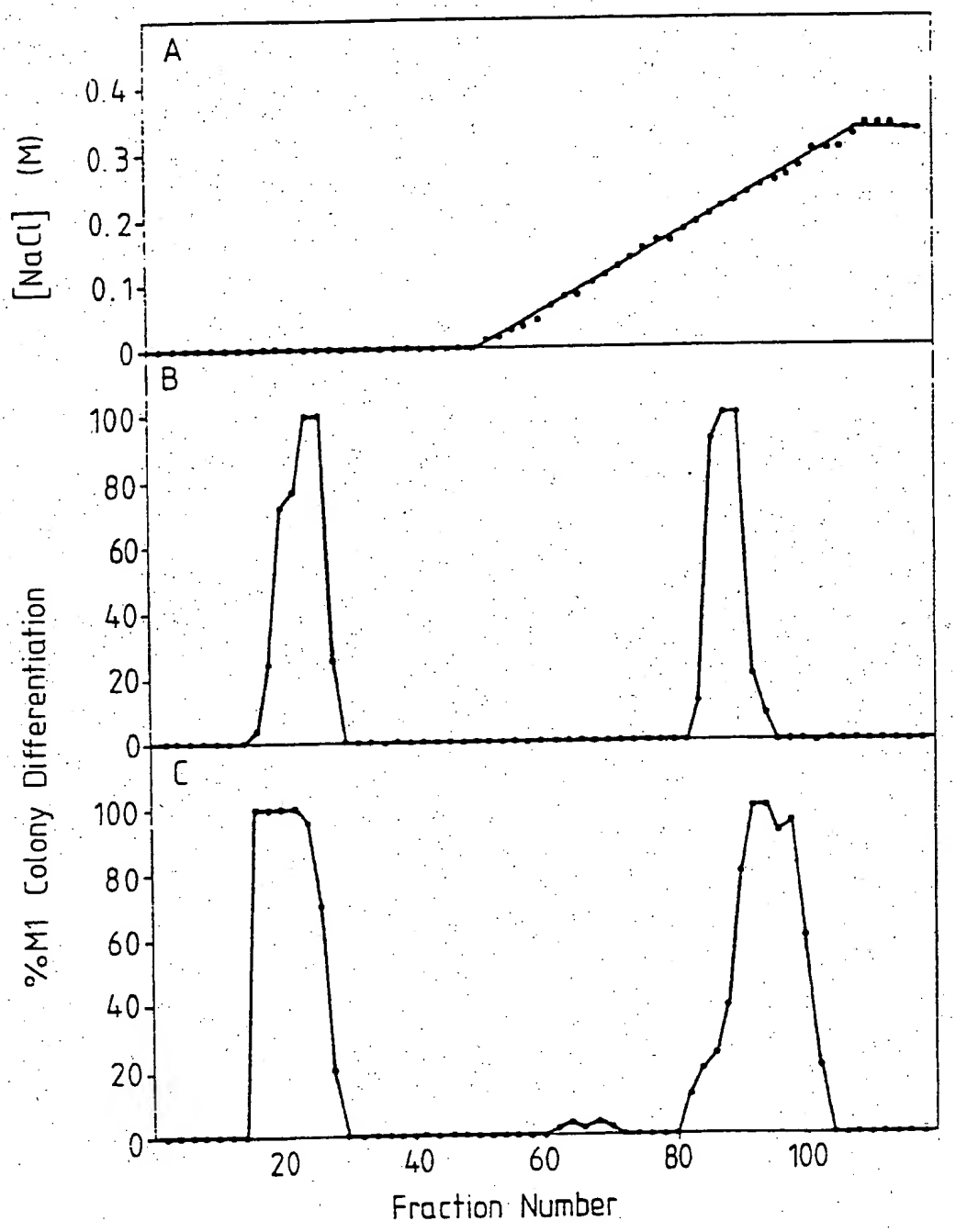

A
B
C
[NaCl] (M)
0.4
0.3
0.2
0.1
0
%M1 Colony Differentiation
100
80
60
40
20
0
20
40
60
80
100
Fraction Number

FIG. 33.

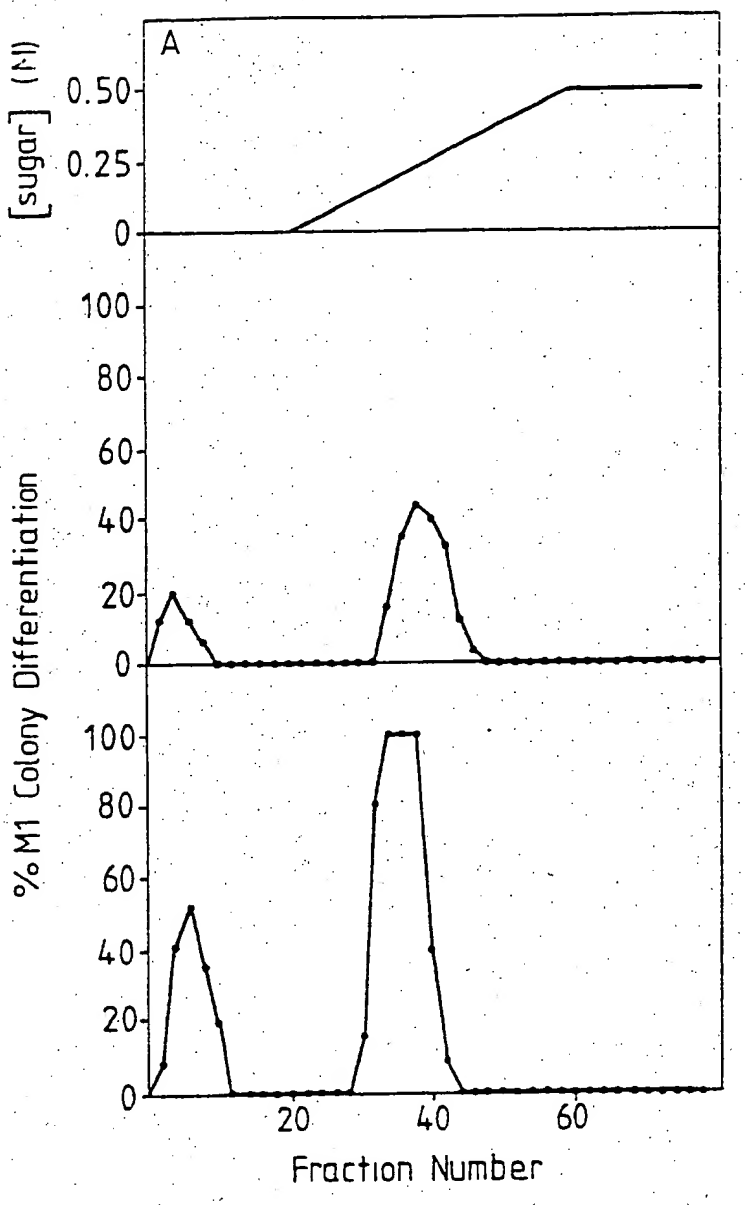

A
[sugar] (M)
0.50
0.25
0
100
80
60
40
20
0
% M1 Colony Differentiation
100
80
60
40
20
0
20
40
60
Fraction Number

FIG. 34.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 88/00093

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07K 13/00,15/12,15/14,C12N 1/18,15/00,C12P 19/34,21/00,21/02, C07H 21/04// (C12P 19/34,C12R 1:91) (C12P 21/02, C12R 1:865)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
WPI, WPIL	(LEUKEMIA or LEUKAEMIA or NEOPLASTIC) and (INHIBIT or INHIBITS or INHIBITING or INHIBITORY) and (FACTORS or PROTEIN or POLYPEPTIDE)
NBRF Database	

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: IPC C07G 7/00, C07K 15/12,14, C12N 15/00, C07H 21/04

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P	AU,A, 66756/86 (HOECHST AKTIENGESELLSCHAFT) 25 June 1987 (25.06.87)	(1-45)
P	Patents Abstracts of Japan, C-482, page 147, JP,A, 62-223126 (SANKYO CO. LTD.) 1 October 1987 (01.10.87)	(1-45)

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 July 1988 (12.07.88)	(10-08-88) 10 AUGUST 1988
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	R. EVANS

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 88/00093

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members			
AU 66756/86	DE 3545568	EP 228018	FI 865186	
	PT 83972	ZA 8609557		

END OF ANNEX

23/kk/48/1